

CONTROL OF INFLAMMATION, HELPER T CELL RESPONSES  
AND REGULATORY T CELL FUNCTION BY BCL6

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## DEDICATION

I would like to dedicate this thesis to my loving parents.

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## ABSTRACT

Deepali Vijay Sawant

### CONTROL OF INFLAMMATION, HELPER T CELL RESPONSES AND REGULATORY T CELL FUNCTION BY BCL6

Regulatory T (Treg) cells represent an important layer of immune-regulation indispensable for curtailing exuberant inflammatory responses and maintaining self-tolerance. Treg cells have translational potential for autoimmunity, inflammation, transplantation and cancer. Therefore, delineating the molecular underpinnings underlying the development, suppressor function and stability of Tregs is particularly warranted. The transcriptional repressor Bcl6 is a critical arbiter of helper T cell fate, promoting the follicular helper (Tfh) lineage while repressing Th1, Th2 and Th17 differentiation. Bcl6-deficient mice develop a spontaneous and severe Th2-type inflammatory disease including myocarditis and pulmonary vasculitis, suggesting a potential role for Bcl6 in Treg cell function. Bcl6-deficient Treg cells are competent in controlling Th1 responses, but fail to control Th2 inflammation in an airway allergen model. Importantly, mice with Bcl6 deleted specifically in the Treg lineage develop severe myocarditis, thus highlighting a critical role for Bcl6 in Treg-mediated control of Th2 inflammation. Bcl6-deficient Tregs display an intrinsic increase in Th2 genes and microRNA-21 (miR-21) expression. MiR-21 is a novel Bcl6 gene target in T cells and ectopic expression of miR-21 directs Th2 differentiation in non-polarized T cells. MiR-21 is up-regulated in mouse models of airway

inflammation and also in human patients with eosinophilic esophagitis and asthma. Thus, miR-21 is a clinically relevant biomarker for Th2-type pathologies. Our results define a key function for Bcl6 in repressing Gata3 function and miR-21 expression in Tregs, and provide greater understanding of the control of Th2 inflammatory responses by Treg cells.

Alexander L. Dent, Ph.D., Chair

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## LIST OF ABBREVIATIONS

Ag	Antigen
Alum	Aluminium hydroxide
APC	Antigen presenting cell
ATL	Angioimmunoblastic T-cell lymphoma
BAL	Bronchoalveolar lavage
BATF	Basic leucine zipper transcription factor ATF-like
Bcl6	B cell lymphoma 6
CD	Cluster of differentiation
ChIP	Chromatin immunoprecipitation
CIA	Collagen induced arthritis
CNS	Conserved non-coding sequence
CTL	Cytotoxic T lymphocyte
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
DC	Dendritic cell
DTR	Diphtheria toxin receptor
EAE	Experimental autoimmune encephalomyelitis
EAU	Experimental autoimmune uveitis
Eomes	Eomesodermin
Foxp3	Forkhead box protein 3
Gata3	Gata binding protein 3
GFP	Green fluorescent protein

HAT	Histone acetyl transferase
HDAC	Histone deacetyl transferase
Hlx	Homeobox-1
ICS	Intracellular cytokine staining
ICOS	Inducible T cell-costimulator
IFN $\gamma$	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
iTregs	Inducible T regulatory cells
JAK	Janus family tyrosine kinases
KO	Knock out
MHC	Major histocompatibility complex
MiRNA	MicroRNA
NFAT	Nuclear factor of activated T cells
NK	Natural killer
NKT	Natural killer T
NOD	Non obese diabetic
nTregs	Natural T regulatory cells
PD-1	Programmed death 1
ROR	Retinoic-acid-related orphan receptor
RORC	Gene name for ROR $\gamma$ t
RT-PCR	Real time polymerase chain reaction

SH2	Src homology 2
SLE	Systemic lupus erythematosus
SOCS3	Suppressor of cytokine signaling 3
STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T cells
Tbx21	Gene name for T-bet
TCR	T cell receptor
Tfh	T follicular helper
Th	T helper
Th1	T helper 1
Th2	T helper 2
Th9	T helper 9
Th17	T helper 17
TGF	Transforming growth factor
TSLP	Thymic stromal lymphopoietin
WT	Wild type
XLP	X-linked lympho-proliferative disease
YFP	Yellow fluorescent protei

## **INTRODUCTION**

### **Innate and Adaptive Immunity**

Immunity is the sophisticated interplay of cells, tissues and mediators that underlie our response to the unfathomable array of pathogens that we are constantly exposed to on a daily basis. The unwavering surveillance and protection conferred by our immune system rarely makes us aware of the diverse microbial challenges that we evade. The ability to discriminate self from non-self and recognize subtle differences between the multitude of infections, generate specific effector responses while curtailing excessive damage to the host and retain memory for subsequent infections constitute the cardinal features of our immune system. The immune system is able to mediate this intricate regulation by the comprehensive defense mechanisms offered by the innate and adaptive arms of the immune system, with each arm functioning independently and in concert with the other to boost the overall immune outcome and provide optimal protection.

The physical and chemical barriers of our body serve as the first line of defense in any infection. Anatomic barriers like the skin and the mucous membranes and physiologic barriers, which include temperature, pH, oxygen tension, and soluble mediators prevent microbes from entering and evading the body. Pathogens that overcome these defense mechanisms activate other components of the innate immune system. The innate immune system, also found in primitive forms of life, acts locally and mobilizes appropriate mediators

for rapid clearance of the infection, although these responses are short-lived and lack immunological memory.

The cells of the innate immune system derived from the myeloid lineage include the tissue-resident macrophages and circulating monocytes, dendritic cells, granulocytes and mast cells. The monocytes that circulate into the blood and their mature tissue forms, i.e. the macrophages constantly patrol the tissues to engulf and phagocytize any invading pathogen or dispose dead cells or components targeted by the adaptive immune system. Macrophages also secrete inflammation promoting components and various signaling mediators in addition to their role as antigen presenting cells, thus serving as an important link between innate and adaptive immunity. Immature dendritic cells are also phagocytic in nature and can engulf particulate matter as well as extracellular fluid by macro-pinocytosis. However, their main function upon maturation is to process and present antigens on their surface for activation of the T cells that bear the appropriate T cell receptor (TCR) and trigger the specific adaptive response. The granulocytes also referred to as polymorphonuclear leukocytes (PMNs) are the major phagocytic cells, distinguished on the basis of their granular contents. Neutrophils increase in large numbers following an infection and they function by phagocytosis and killing mediated by the oxidative and antimicrobial mediators stored in their granules. Eosinophils and basophils direct protective responses against parasites, which are difficult to engulf by the macrophages or neutrophils. Excessive eosinophilic activity however is associated with allergic responses (Wardlaw *et al.*, 2000). Basophils and their



inflammatory mediators like histamine and leukotrienes are also associated with allergy, and more recently, basophils have been identified as a primary source of the Th2 cytokine, IL-4 (Min *et al.*, 2004). Mast cells differentiate in tissues and act locally to release inflammatory mediators in allergic responses and during parasitic worm clearance.

The lymphoid components of the innate immune system comprise NK cells, NKT cells and gamma-delta T cells. NK cells are cytotoxic lymphocytes that lack antigen-specific receptors. They are primarily responsible for killing virus-infected cells and tumor cells. NKT cells exhibit features intermediate of NK cells (cell surface expression of NK1.1) and T cells (TCRs recognize lipid antigens presented by CD1d molecules), hence generally referred to as CD1d-restricted T cells. They function by secreting cytokines like IFN $\gamma$ , IL-4, TNF $\alpha$  and GM-CSF. These cells are known for recognizing glycolipids from pathogens such as *Mycobacterium tuberculosis* and are also implicated in autoimmunity and cancer (Bendelac *et al.*, 2007). Gamma–delta T cells ( $\gamma\delta$  T) express a TCR receptor comprised of  $\gamma$  and  $\delta$  chains, unlike conventional  $\alpha\beta$  T cells. Although capable of generating more TCR repertoire diversity than  $\alpha\beta$  T cells, these cells tend to have restricted TCR repertoires. Despite the presence of functional TCRs, these cells are at the border between innate and adaptive immunity, primarily because their TCR can also function as a pattern recognition receptor and in some cases, they have been ascribed a phagocytic function (Morita *et al.*, 2000). Like NKT cells, these cells primarily recognize lipid antigens and are thus useful for

clearance of bacterial and viral infections, but also have clear roles in autoimmunity, hypersensitivity and tumor immunity (Cai and Tucker, 2001).

The innate immune response is often overlapped by adaptive immunity, which sets in days after infection, but provides a more directed and lasting response, retaining memory for subsequent encounters. This system is present only in higher forms of life and functions due to the specificity of the antigen receptors characteristic of its principal components, i.e. B and T lymphocytes. B and T lymphocytes both develop in the bone marrow. Following maturation in the bone marrow (for B cells) and thymus (for T cells), these 'naïve' T cells circulate in the body, bearing a highly specific receptor on their surface. Following interaction with the cognate antigen, these cells get activated into effector lymphocytes. In the case of B cells, antigen recognition through the BCR leads to generation of memory B cells and plasma cells. Plasma cells secrete antibodies with the same antigenic specificity as the surface BCR, thus conferring humoral immunity to the specific pathogen. TCR triggering of immature thymic T cells generates either effector cytotoxic CD8 or helper CD4 T cells or regulatory T cells. Memory B and T cells survive longer to provide lasting immunity for subsequent exposures. Cytotoxic CD8 T cells mediate direct killing of the virus-infected or cancer cells by recognizing the antigen complexed with an MHC Class I molecule. Their killing is directed by release of cytolytic proteins such as granzyme and perforin or by up-regulating Fas expression on their surface and thus triggering death of the FasL-expressing target cell. Cytotoxic T cells are protective in a number of autoimmune diseases such as diabetes, arthritis and

liver injury following viral infections (Pender, 2012). Helper CD4 T cells recognize antigen in the context of Class II MHC and differentiate into diverse subtypes based on the cytokine cues and provide help in a number of inflammatory and infectious settings, in addition to providing specialized help to B cells for protective humoral responses.

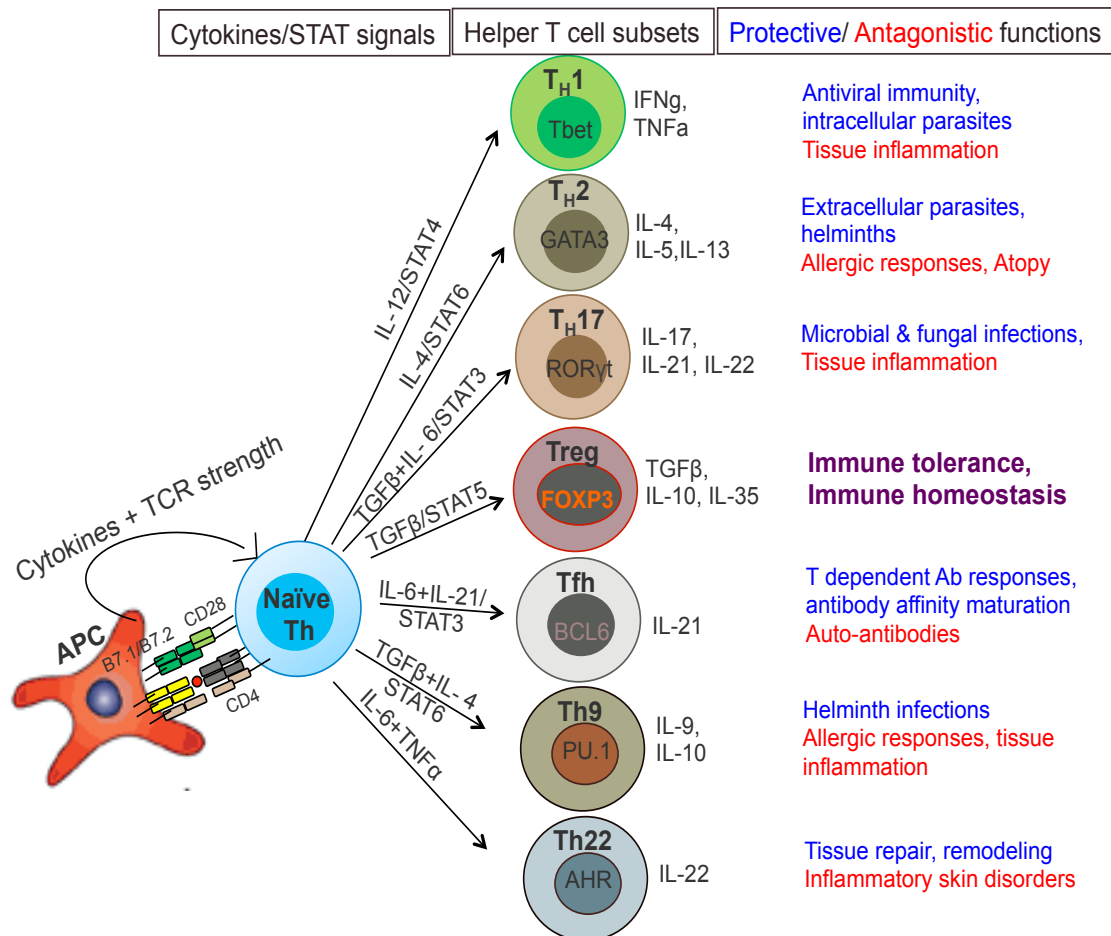
### **Helper T cell subsets**

Following recognition of a cognate antigen delivered by professional antigen-presenting cells (APCs) in the context of MHC class II, naïve T cells have the potential to differentiate into various helper CD4 T cell subsets. Priming of naïve T cells by APCs is mediated by three distinct signals that are required for optimal activation and differentiation to specialized helper CD4 T cell lineages specialized to combat different types of pathogens. Signal 1 comprises recognition of a specific peptide-MHC complex on the APC with the T cell receptor. Signal 2 referred to as co-stimulation is essential for survival and clonal expansion of antigen-specific T cells. Antigen recognition in the absence of co-stimulation can lead to functional inactivation or deletion of the activated T cells. The decision to differentiate into a specific T helper cell subset is provided by the cytokines present in the local milieu (Signal 3), particularly, those made by the priming APCs in response to the pathogenic insult. Most cytokines signal through the JAK/STAT pathway linking receptor-associated Janus kinases (JAKs) to the Signal Transducer and Activators of Transcription (STAT) family of transcription factors. Cytokine-mediated engagement of the receptor triggers activation of

JAKs and phosphorylation of the receptor cytoplasmic domains, providing docking sites for STATs. Phosphorylation of the STATs by JAKs allows their dimerization and translocation to the nucleus, where they function as transcription factors to initiate a specific gene expression program. There are four members in the JAK family (Jak1, Jak2, Jak3 and Tyk2) and seven STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6). The differentiation of helper T cells to different subsets is mediated by specific cytokines, activating a specific set of JAK and STAT members that ultimately dictates their effector functions (Figure 1).

The initial paradigm of helper T cell differentiation was the description of Th1 and Th2 subsets by Mossman and Coffman in 1986 (Mosmann *et al.*, 1986), with Th1 and Th2 cells being critical for immunity to intracellular and extracellular pathogens, respectively. These initial studies provided a well-defined framework to understand mechanisms of gene regulation and cell fate specification and gave enormous insights into T cell responses to diverse microbial pathogens. However, the Th1/Th2 paradigm failed to explain several observations pertaining to immunity and autoimmune responses. This led to the eventual discovery of additional subsets – Th17, regulatory T cells (Tregs), Th9, Th22 and Tfh cells. Functional specialization to these different helper T cell subsets is achieved through differential expression of lineage-defining transcription factors and cytokine signatures that are both counter-regulatory and self-reinforcing in nature. These intrinsic and extrinsic cues guide the development of stable T

helper cell lineages that are crucial for proper immune-regulation and host defense.



**Figure 1: Helper T cell differentiation program is guided by an orchestrated interplay of priming cytokines and corresponding STAT family members.**

Naïve T cells differentiate into various helper T cell subsets based on the cytokines secreted by the priming antigen-presenting cells (APCs) and the strength of T cell signals. Cytokine signaling activates the corresponding JAK/STAT pathway to initiate the transcriptional program, with expression of specific transcription factors and cytokines key to each subset, that underlie their protective and antagonistic effects in diverse immune settings.

## Th1 cells

Th1 cells are primarily responsible for protective immunity to intracellular pathogens (Hsieh *et al.*, 1993; Mosmann and Coffman, 1989; Zhu and Paul, 2008). In humans, Th1 cells are particularly important for resistance to mycobacterial infections. Priming of a naïve CD4<sup>+</sup> T cell precursor to the Th1 lineage is driven by IL-12 produced by macrophages and dendritic cells. IL-12-induced activation of STAT4 is critical for Th1 development (Hsieh *et al.*, 1993; Kaplan *et al.*, 1996b). Commitment to the Th1 lineage is further enhanced by activation of STAT1 by IFN $\gamma$  made by innate immune system cells such as NK cells, macrophages, and dendritic cells. The master Th1 transcription factor, T-bet (*Tbx21*) is induced upon TCR stimulation and downstream of IFN $\gamma$ /STAT1 activation (Lighvani *et al.*, 2001; Szabo *et al.*, 2000). T-bet is expressed preferentially in Th1 cells and forced expression of T-bet in committed Th2 cells can also skew them towards Th1 fate (Mullen *et al.*, 2001; Szabo *et al.*, 2000). T-bet induces IFN $\gamma$  expression in T cells and also up-regulates IL-12R $\beta$ 2, thereby increasing the responsiveness of the developing Th1 cells to IL-12/STAT4 signaling leading to production of more IFN $\gamma$ . Thus the collaboration between IL-12 and IFN $\gamma$ -mediated signaling events is required for full commitment to the Th1 lineage (Murphy and Reiner, 2002). IL-18 has also been reported to act in combination with IL-12 to up-regulate IFN $\gamma$  in Th1 cells. Thus, mice deficient in components of the Th1 differentiation pathway (IL-12, IL-12 receptor, STAT1, STAT4, IFN $\gamma$  and IL-18) exhibit defective Th1 responses to bacterial infections (Meraz *et al.*, 1996; Tarleton *et al.*, 2000). Transcription factors homeobox-1 (Hlx)

(Mullen *et al.*, 2002), Runx3 (Djuretic *et al.*, 2007; Kohu *et al.*, 2009) and ERM (Ouyang *et al.*, 1999) are expressed in Th1 cells and have a role in IFN $\gamma$  production in Th1 cells. The Th2 transcription factor, Gata3, has been demonstrated to down-regulate both IFN $\gamma$  and IL-12R $\beta$ 2 expression (Lee *et al.*, 2000; Ouyang *et al.*, 1998). Similarly, the Th2 cytokine, IL-4 has also been shown to down-regulate IL-12R $\beta$ 2 expression on developing cells, thereby skewing them towards Th2 fate.

The signature cytokines for the Th1 lineage are IFN $\gamma$ , IL-2 and lymphotoxin alpha (de Jong *et al.*, 1998). IFN $\gamma$  is important to increase the microbicidal activity of macrophages (Suzuki *et al.*, 1988). IL-2 is important for CD4<sup>+</sup> T cell memory; IFN $\gamma$ <sup>+</sup>IL-2<sup>+</sup> CD4 T cells are considered as precursors of Th1-memory cells (Darrah *et al.*, 2007). Together with IL-2, IFN $\gamma$  promotes differentiation of CD8 T cells into actively cytotoxic lymphocytes (Williams *et al.*, 2006). LT $\alpha$  is involved in activation and recruitment of effector cells to infection sites and is implicated as tissue damage factor in autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (Selmaj *et al.*, 1991; Suen *et al.*, 1997). Th1 cells support the production of opsonizing antibodies (predominantly IgG). Chemokine receptors CXCR3 and CCR5 are preferentially expressed on Th1 cells and mediate homing of these cells to sites of infection (Bonecchi *et al.*, 1998; Sallusto *et al.*, 1998). Inappropriate Th1 responses have been implicated in mouse models of a number of autoimmune and inflammatory pathologies (experimental autoimmune encephalomyelitis, EAE; experimental autoimmune uveitis, EAU; T cell transfer models of colitis) (Caspi *et al.*, 1996; Fuss *et al.*,

1999; Germann *et al.*, 1996; Merrill *et al.*, 1992). Polymorphisms in the gene encoding T-bet (*Tbx21*) have been associated with increased incidence of asthma and airway hyper-responsiveness (Raby *et al.*, 2006). Mutations in IL-12R $\beta$ 1 in humans have been linked with increased susceptibility to mycobacterial and salmonella infections (de Jong *et al.*, 1998), while mutations in the human IFN $\gamma$  or IFN $\gamma$ R1 genes are associated with increased susceptibility to intracellular infections (Newport *et al.*, 1996).

## **Th2 cells**

Th2 cells mediate host defense against extracellular parasites, including helminth infections, and are critical for protective humoral immune responses (Paul and Seder, 1994; Zhu and Paul, 2008). Priming of naïve T cells by IL-4 activates STAT6, which is required for Th2 cell development (Kaplan *et al.*, 1996a; Shimoda *et al.*, 1996). STAT6 activation is both necessary and sufficient to induce the Th2 lineage master transcription factor, Gata3 (Kurata *et al.*, 1999; Zhu *et al.*, 2001). Gata3 then activates transcription of the characteristic Th2 cytokines, IL-4, IL-5, IL-13, and can also auto-activate its own expression (Ouyang *et al.*, 1998). Ectopic expression of Gata3 in Th1 lineage induces IL-4 production. At the same time, deletion of Gata3 in Th2 cells blocks IL-5 and IL-13 production, but has modest effects on IL-4, consistent with the presence of Gata3 binding sites in the IL-5 and IL-13 promoters (Zhu *et al.*, 2004). Along with the IL-4-induced activation of Gata3, the gamma-chain cytokines (IL-2, IL-7 and TSLP)-driven activation of STAT5 is required to cooperate with Gata3 for full Th2



differentiation (Zhu *et al.*, 2003). Other transcription factors, STAT3, cMAF and IRF4 are also expressed in Th2 cells and are required for Th2 differentiation (Kim *et al.*, 1999; Lohoff *et al.*, 2002; Stritesky *et al.*, 2011). Gfi-1 is an IL-4 inducible factor that allows for the preferential expansion of Gata3-expressing Th2 cells (Zhu *et al.*, 2006). Th2 differentiation is strongly potentiated by a positive feedback loop involving autocrine activation of IL-4 transcription by exogenous IL-4 and the ability of Gata3 to auto-activate its own transcription. Th2 responses in mice deficient in the Th2 inhibitory transcription factor Bcl6 occur in an IL-4/STAT6 independent manner, highlighting a STAT6-independent pathway of Th2 differentiation (Dent *et al.*, 1999). Ectopic expression of the Th1 transcription factor, T-bet in developing T cells promotes down-regulation of IL-2 and Th2 cytokines, demonstrating antagonistic regulation of Th2 differentiation by Th1 pathway (Usui *et al.*, 2006).

Th2 cells produce IL-4, IL-5, IL-9, IL-10, IL-13, IL-25 and amphiregulin. IL-4 is the key positive feedback cytokine for Th2 differentiation and along with IL-13 is required for class switching to IgE in B cells and alternative macrophage activation (Kopf *et al.*, 1993). IL-5 is required for eosinophil recruitment, proliferation and survival (Coffman *et al.*, 1989), IL-9 promotes mast cell activation and induces mucin production in epithelial cells during allergic reactions (Longphre *et al.*, 1999), IL-10 suppresses Th1 cell proliferation (Moore *et al.*, 2001), and IL-13 mediates airway hyper-responsiveness, mucus secretion and is important in helminth expulsion (Wynn, 2003). IL-25 functions as an initiating and amplifying factor for Th2 responses and also induces the production

of eosinophil recruiting chemokines, RANTES (CCL5) and eotaxin (CCL11) (Fort *et al.*, 2001). Amphiregulin induces epithelial cell proliferation and is important to restore epithelial integrity and tissue homeostasis (Zaiss *et al.*, 2006). Chemokine receptors CCR3, CCR4, CCR8 and CRTh2 are preferentially expressed on Th2 cells (D'Ambrosio *et al.*, 1998; Sallusto *et al.*, 1997). Exaggerated Th2 responses are associated with allergic reactions to environmental and food antigens and also airway inflammation and hyper-responsiveness in asthmatic individuals (Holgate, 1999). Individuals with Gata3 haploinsufficiency develop hypoparathyroidism, sensorineural deafness, and renal dysplasia (HDR) syndrome (Van Esch *et al.*, 2000). Polymorphisms in Gata3 in Finnish populations are associated with elevated IgE and increased susceptibility to asthma (Pykalainen *et al.*, 2005). Variants of IL4R $\alpha$  have been linked to atopic asthma, elevated IgE and severe atopic dermatitis (Hershey *et al.*, 1997).

### **Th17 cells**

Th17 cells are important for host protection from extracellular bacteria and fungal infections (Weaver *et al.*, 2006). The discovery of IL-23 in the early 2000s called for reassessment of the relative contribution of IL-12 and IL-23 in the pathogenesis of experimental autoimmune encephalomyelitis (EAE) to explain the differential disease outcomes with deficiency of IL-12 and IL-23 (since both cytokines share the IL12p40 subunit) (Cua *et al.*, 2003). These studies led to the identification of Th17 cells, as a separate lineage from Th1/Th2 cells, that are key

players in the pathogenesis of many organ-specific autoimmune diseases, initially considered to be dependent on Th1 cells (Harrington *et al.*, 2005; Park *et al.*, 2005). Naïve T cells activated in the presence of IL-6 and TGF $\beta$  promote Th17 development (Bettelli *et al.*, 2006; Mangan *et al.*, 2006; Veldhoen *et al.*, 2006). IL-6 driven activation of STAT3, up-regulates the Th17 master transcription factor, ROR $\gamma$ t (Ivanov *et al.*, 2006). Ectopic expression of ROR $\gamma$ t induces IL-17 production in T cells. IL-21, a cytokine also produced by Th17 cells can replace IL-6 to induce ROR $\gamma$ t and IL-17 expression (Korn *et al.*, 2007a; Nurieva *et al.*, 2007). IL-23, another STAT3-inducing cytokine, is not required during Th17 priming, but is critical for survival and maintenance of the Th17 fate (Stritesky *et al.*, 2008; Veldhoen *et al.*, 2006). Thus, differentiation to the Th17 lineage is dependent on three steps: Differentiation stage mediated by the innate immune cell derived Th17-promoting cytokines, IL-6 and TGF $\beta$ ; amplification step driven by IL-21, and phenotype maintenance stage mediated by IL-23. Since IL-6, IL-21 and IL-23 signal via STAT3, STAT3 is indispensable for Th17 differentiation (Harris *et al.*, 2007; Mathur *et al.*, 2007). Other transcription factors – ROR $\alpha$ , cMAF and IRF4 are up-regulated in Th17 cells and required for optimal Th17 differentiation (Brustle *et al.*, 2007; Hiramatsu *et al.*, 2010; Yang *et al.*, 2008b). The aryl hydrocarbon receptor is highly expressed in Th17 cells and is important for production of IL-22 (Veldhoen *et al.*, 2008a). Th1 cytokine, IFN $\gamma$  and Th2 cytokine, IL-4 negatively regulate Th17 differentiation (Harrington *et al.*, 2005; Veldhoen *et al.*, 2008a). IL-2 induced STAT5 can compete with STAT3 and hence is also an inhibitory cytokine for Th17 cells (Laurence *et al.*, 2007; Yang *et*

*et al.*, 2011). In addition, IL-2 is important for Foxp3 induction and Treg development, which is antagonistic to Th17 differentiation program (Burchill *et al.*, 2007b; Davidson *et al.*, 2007).

Th17 cells produce IL-17A, IL-17F, IL-21 and IL-22. Both IL-17A and IL-17F are important for neutrophil recruitment during protective responses to extracellular bacteria and fungi. In addition to its role as a positive feedback cytokine in Th17 amplification phase, IL-21 also has functions in CD8 T cells, B cells and DCs (Leonard and Spolski, 2005). IL-22 is important for immunity to bacteria such as *Klebsiella pneumoniae* and *Citrobacter rodentium* (Aujla *et al.*, 2008). IL-22 protects hepatocytes during liver inflammation and mediates IL-23 dependent skin inflammation (Zenewicz *et al.*, 2007). Th17 cells preferentially express CCR6 and CCR4 (Acosta-Rodriguez *et al.*, 2007). IL-17A also induces other pro-inflammatory cytokines like IL-6 and is implicated in several organ-specific autoimmune diseases like experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA) and mouse models of type1 diabetes (Emamaullee *et al.*, 2009; Komiyama *et al.*, 2006; Nakae *et al.*, 2003). Pathogenic Th17 cells have been implicated in human patients with autoimmune disease such as psoriasis, multiple sclerosis, type1 diabetes and rheumatoid arthritis. Individuals with Hyper-immunoglobulin E or Job's Syndrome (dominant negative mutations in STAT3) show increased susceptibility to staphylococcal and fungal infections due to inability to develop Th17 cells (Holland *et al.*, 2007; Milner *et al.*, 2008). Patients with chronic mucocutaneous candidiasis (CMC) exhibit either an IL17RA autosomal recessive deficiency or IL17F autosomal

dominant deficiency and are subject to chronic infections with *Candida albicans* and *S. aureus* (Puel *et al.*, 2011). Clinical trials with neutralizing antibody to IL-17A have been successful in treating psoriasis, rheumatoid arthritis, and multiple sclerosis (Genovese *et al.*, 2010; Leonardi *et al.*, 2012). However, exacerbations noted in Crohn's disease patients has cast doubt on universal pro-inflammatory nature of Th17 cells and led to identification of regulatory Th17 (rTh17) cells in the gut that are important for mucosal tolerance (Esplugues *et al.*, 2011; Sandborn *et al.*, 2008).

### **Tfh cells**

The concept of T cell-help to B cells for protective humoral immune responses was known for over 30 years, but the exact nature of these helper T cells remained controversial. Progress in recent years in trying to characterize the cell surface markers associated with this helper T cell lineage has led to the identification of T follicular helper (Tfh) cells as a distinct cell lineage separate from Th1, Th2 and Th17 cells (Chtanova *et al.*, 2004; Kim *et al.*, 2001; King *et al.*, 2008; Vinuesa *et al.*, 2005). Tfh cells are characterized by increased expression of the B cell follicle homing receptor CXCR5 (Breitfeld *et al.*, 2000; Schaerli *et al.*, 2000), the co-stimulatory molecule, ICOS (Bauquet *et al.*, 2009; Choi *et al.*, 2011) and the inhibitory receptor, PD-1 (Haynes *et al.*, 2007). *In vitro*, Tfh cells differentiate in the presence of STAT3-inducing cytokines IL-6 and IL-21 (Chtanova *et al.*, 2004; Nurieva *et al.*, 2008). These factors induce the transcriptional repressor Bcl6 that has been demonstrated as the lineage-

defining factor for Tfh cells by three independent groups in 2009 (Johnston *et al.*, 2009; Nurieva *et al.*, 2009; Yu *et al.*, 2009). Bcl6 is both required and sufficient for Tfh differentiation as shown by complete lack of Tfh cells in Bcl6-deficient mice and ectopic expression of Bcl6 capable of up-regulating hallmark Tfh genes – CXCR5, CXCR4, PD-1, ICOS, IL-6R, IL-21R and down-regulating CCR7, both *in vitro* and *in vivo* (Yu and Vinuesa, 2010). Bcl6 expression in T cells is important for CXCR5 expression, T cell migration into follicles and germinal center formation. Bcl6 directs Tfh differentiation by (1) repressing Th1, Th2 and Th17 differentiation of T cells, by direct binding or inhibiting the function of their transcriptional regulators (Nurieva *et al.*, 2009; Yu *et al.*, 2009), (2) antagonistic regulation of BLIMP1, which inhibits Tfh differentiation and promotes differentiation to effector T cell lineages (Johnston *et al.*, 2009) and/or by repressing miRNAs that target Tfh genes (eg; Bcl6 represses miR-17-92 that targets CXCR5 and miR101-103 that targets ICOS) (Yu *et al.*, 2009). Expression of CXCR5 allows Tfh cells to home to the B cell follicles where the CXCR5 ligand, CXCL13 is expressed (Ansel *et al.*, 1999). Although expression of neither CXCR5, Bcl6, ICOS or IL-21 is an exclusive feature of Tfh cells, cells that are CXCR5<sup>high</sup>, ICOS<sup>high</sup>, PD-1<sup>high</sup>, express high levels of Bcl6 and low levels of T-bet, Gata3, RORγt, and Foxp3 and secrete high levels of IL-21 are best defined as Tfh cells (Yu and Vinuesa, 2010). IL-21 promotes Tfh cell survival in an autocrine manner and is also important for GC B cell survival, affinity maturation and differentiation (Linterman *et al.*, 2010; Vogelzang *et al.*, 2008; Zotos *et al.*, 2010). However, both IL-6 and IL-21 alone can induce Tfh cells, *in vivo* and *in vitro*,

indicating redundancy in the actions of these two STAT3 activating cytokines in Tfh differentiation (Eto *et al.*, 2011; Poholek *et al.*, 2010). Loss of Tfh cells was reported in CD4-specific STAT3 deficient mice, however, another study showed the presence of CXCR5<sup>+</sup> Tfh cells in a STAT3-deficient setting and STAT3 seems dispensable for human Tfh differentiation (Eddahri *et al.*, 2009; Schmitt *et al.*, 2009). IL-12 acting via STAT4 has been reported to induce Tfh genes, Bcl6 and IL-21 (Ma *et al.*, 2009; Nakayamada *et al.*, 2011; Schmitt *et al.*, 2009). Thus, STAT4 may replace STAT3 functions in terms of Tfh differentiation. The transcription factor cMAF regulates IL-4 and IL-21 production, which are major cytokines made by GC Tfh cells (Bauquet *et al.*, 2009; Kroenke *et al.*, 2012). cMaf-deficient mice have defective Tfh differentiation *in vivo*. Mice deficient in BATF have impaired Tfh development, defects in GC formation and B cell class-switch recombination (Betz *et al.*, 2010; Schraml *et al.*, 2009). BATF mediates these effects via intrinsic functions in both B and T cells, and also binds to the IL-21 promoter. Mice lacking IRF4 are also deficient in Tfh cells (Kwon *et al.*, 2009). Since Bcl6 and IRF4 are mutually antagonistic factors in GC B cells (Saito *et al.*, 2007) and IRF4 can induce the Tfh antagonistic factor BLIMP1 in CD4 T cells (Kwon *et al.*, 2009), the exact mechanism for the role of IRF4 in Tfh cells needs further investigation.

Subsets of patients with common variable immunodeficiency (CVID) lack ICOS and these patients lack memory B cells and Tfh cells, which may be associated with increased susceptibility to various bacterial, viral and fungal infections and lack antigen-specific IgG responses to vaccines (Bossaller *et al.*,

2006; Grimbacher *et al.*, 2003; Warnatz *et al.*, 2006). Individuals with X-linked lympho-proliferative disease (XLP) have mutations in *Sh2d1a* (which encodes SAP) have increased susceptibility to childhood infections, particularly, Epstein-Barr virus (EBV). This is due to extreme loss of GCs and memory B cells in these patients (Ma *et al.*, 2006; Malbran *et al.*, 2004). Increased Tfh activity, dysregulated GCs and autoantibodies have been reported in a number of mouse models of autoimmune diseases like systemic lupus erythematosus (SLE), rheumatoid arthritis and diabetes (Hutloff *et al.*, 2004; Kendall *et al.*, 2007; Luzina *et al.*, 2001; Weyand and Goronzy, 2003). In addition, spontaneous GCs are typical in spontaneous models of lupus, insulinitis and arthritis (namely MRL, MRL/lpr, Sanroque, NOD and K/BxN) (Hutloff *et al.*, 2004). Circulating CD4<sup>+</sup>CXCR5<sup>+</sup> Tfh cells are present in humans with systemic autoimmunity and this population is expanded in 20-30% patients with lupus and Sjogren's syndrome (Kang *et al.*, 2011; Simpson *et al.*, 2010). Tfh cells may also be involved in lymphomagenesis as indicated by presence of Tfh-like cells (Bcl6<sup>+</sup>, PD-1<sup>+</sup>, ICOS<sup>+</sup>) in patients with angioimmunoblastic T-cell lymphoma (ATL) (Ellyard *et al.*, 2012).

### **Th9 cells**

Latest addition to the expanding genre of helper T cell subsets include Th9 cells and Th22 cells that secrete IL-9 and IL-22 as signature cytokines, respectively. Th9 cells differentiate *in vitro* following treatment of naïve CD4<sup>+</sup> T cells with IL-4 and TGFβ or by culturing Th2 cells in the presence of TGFβ



(Dardalhon *et al.*, 2008; Veldhoen *et al.*, 2008b). Unlike Th2 cells, Th9 cells secrete high IL-9 and reduced levels of Th2 cytokines (IL-4, IL-5 and IL-13) (Chang *et al.*, 2010). The role of TGF $\beta$  in inducing IL-9 production was originally reported in 1994 by Schmitt *et al.*, who also reported that IL-4 could potentiate and IFN $\gamma$  could inhibit this effect (Schmitt *et al.*, 1994). Both IL-4 and TGF $\beta$ -induced transcription factors and signaling cascades are important for optimal IL-9 production, as cells deficient in either component fail to differentiate into Th9 lineage (Dardalhon *et al.*, 2008; Goswami *et al.*, 2012; Veldhoen *et al.*, 2008b). Signaling via TGF $\beta$  induces PU.1, an ETS-family transcription factor that is key to the development of Th9 cells (Chang *et al.*, 2010). PU.1 is preferentially expressed in Th9 cells, wherein PU.1 negatively regulates Th2 cytokine production by interfering with the interaction of IRF4 and Gata3 (Chang *et al.*, 2009; Chang *et al.*, 2010; Chang *et al.*, 2005). Pu.1-deficient Th9 cells secrete reduced IL-9 levels, while ectopic PU.1 expression rescues production of IL-9, partially by direct binding to the IL-9 promoter (Chang *et al.*, 2010). IL-4 acting via STAT6 functions indirectly in priming Th9 differentiation, by counteracting the negative regulation provided by Foxp3 and T-bet in Th9 cells (Goswami *et al.*, 2012). In addition, IL-4 and STAT6 also induce the expression of other transcription factors (Gata3, IRF4, cMAF) that positively regulate Th9 fate (Goswami *et al.*, 2012; Jabeen and Kaplan, 2012).

The hallmark Th9 cytokine, IL-9, initially considered as a Th2 cytokine implicated in protective immunity to helminth infections, has been shown to promote allergic responses (Faulkner *et al.*, 1998; Khan *et al.*, 2003). IL-9

promotes inflammation by acting as a survival factor for mast cells, and by secreting mediators like chemokines that recruit cells to sites of inflammation (Goswami and Kaplan, 2011; Neill and McKenzie, 2010). Pathogenic potential of Th9 cells has been demonstrated in a number of mouse models of autoimmune diseases, namely T cell transfer model of colitis (Dardalhon *et al.*, 2008), EAE (Jager *et al.*, 2009), ocular inflammation (Tan *et al.*, 2010) and allergic airway inflammation (Chang *et al.*, 2010; Cheng *et al.*, 2002; Temann *et al.*, 2007). Increased IL-9 levels have also been reported in stimulated PBMCs from atopic children and BAL of asthmatic patients compared to healthy controls (Chang *et al.*, 2010; Erpenbeck *et al.*, 2003).

## **Th22 cells**

Naïve human CD4<sup>+</sup> T cells differentiate into Th22 cells *in vitro* in the presence of IL-6 and TNF $\alpha$  (Eyerich *et al.*, 2009; Trifari *et al.*, 2009). Similar to Th17 cells, Th22 cells express IL-22, CCR4, and CCR6, but in contrast, they also express CCR10 and several fibroblast growth factors (FGFs). In addition, Th22 cells do not express IL-17, CCL20, IL-23R, CD161 (Th17 markers), IL-4 (Th2 marker), or IFN-gamma (Th1 marker). Collectively, these characteristics distinguish Th22 cells as a novel T helper cell lineage that is distinct from the Th17, Th2, and Th1 subtypes (Eyerich *et al.*, 2009; Trifari *et al.*, 2009). The ligand-dependent transcription factor Aryl hydrocarbon receptor (Eddahri *et al.*) regulates the expansion of the IL-22 expressing T cells (Ramirez *et al.*, 2010; Trifari *et al.*, 2009). RNAi-mediated knockdown of AHR in human memory T cells

reduced IL-22 production from these cells, whereas IL-17 was not affected (Trifari *et al.*, 2009). Similarly, Th17 cells from Ahr-deficient mice failed to produce IL-22 (Veldhoen *et al.*, 2008a). The cytokine IL-22, an IL-10 family member (Wolk *et al.*, 2010), is a unique cytokine produced by immune cells - Th1, Th17, gamma-delta T cells but CD4<sup>+</sup>Th22, IL-22 producing CD8 T and innate lymphoid cells (LTi and NK22) are the predominant producers of IL-22 (Trifari and Spits, 2010). IL-22 bridges the immune and the non-hematopoietic stromal compartment by acting only on epithelial cells, keratinocytes and myofibroblasts, which express IL-22R. Expression of the CCR4 and CCR10 skin-homing receptors on Th22 cells suggests that these cells are likely recruited to the skin, where they may contribute to host defense against microbial pathogens, and promote tissue repair or remodeling (Pickert *et al.*, 2009; Zheng *et al.*, 2007a). IL-22 is up-regulated in rheumatoid arthritis, crohn's disease, psoriasis and atopic dermatitis patients, whereas it is down-regulated in the sera of patients with sarcoidosis and systemic lupus erythematosus (Zhang *et al.*, 2011). Multiple studies indicate that Th22 cells may also be involved in the pathogenesis of inflammatory skin disorders such as psoriasis, atopic eczema, and allergic contact dermatitis (Eyerich *et al.*, 2009; Fujita, 2012; Vollmer *et al.*, 1994).

### **Treg cells**

The immune system has developed elaborate mechanisms to enable effective immune responses against the inestimable number and types of pathogenic challenges. These responses are possible due to the diversity of

lymphocyte antigen receptors generated in the process of somatic gene rearrangements. While this process of random gene rearrangements prepares the host to respond to the rapidly evolving infectious agents, it also creates the danger of mounting harmful immune responses to self-components. Cell-intrinsic mechanisms of recessive tolerance that ensure the deletion or functional inactivation of self-reactive T cells are operative both in the thymus and periphery. However, these mechanisms are often insufficient and perfected by complementary dominant tolerance mediated by regulatory T cells acting in *trans* (Josefowicz *et al.*, 2012a). The initial effector phase in any immune response is followed by a shift to the regulatory subset that limits the exaggerated response and balances the immune outcome, without excessive damage to the host. While there are other suppressive lymphoid subsets, regulatory T cells (Tregs) represent a major dedicated immune-regulatory subset of CD4 T cells, essential for dominant control of peripheral tolerance and maintenance of immune homeostasis.

### **Identification of “suppressor T cells”**

The earliest evidence for the presence of a “suppressor” T cell population was noted in studies wherein thymectomy of neonatal mice between 2 and 4 days of age lead to a fatal autoimmune disease, that could be prevented by adoptive transfer of thymocytes or splenocytes from adult euthymic mice (Asano *et al.*, 1996; Bonomo *et al.*, 1995; Nishizuka and Sakakura, 1969). This suggested that a suppressor T cell population emerged from the thymus after 3

days that controlled the development of autoimmunity. Chicken-quail chimera transplantation experiments suggested that grafted thymic epithelium was necessary for xenograft tolerance, thus implicating a population of thymus-derived suppressor cells in the control of allo-reactivity (Ohki *et al.*, 1987). These initial autoimmunity and transplant tolerance studies culminated with the identification of a subset of CD4 T cells that constitutively expressed the IL-2R alpha chain (CD25), referred as regulatory T cells or Tregs, by Sakaguchi and colleagues in 1995 (Sakaguchi *et al.*, 1995). Transfer of CD4 T cells depleted of the CD25<sup>+</sup> population into athymic nude mice triggered an autoimmune inflammatory pathology, but transfer of intact CD4 T cell population did not. These CD4<sup>+</sup>CD25<sup>+</sup> T cells were able to limit inflammation in models of autoimmunity and transplant rejection (Fehervari and Sakaguchi, 2004; Shevach, 2000). However, the presence of CD25 on activated T cells impeded efforts in distinguishing these suppressor cells from activated inflammatory CD4<sup>+</sup> T cells.

### **Treg transcription factor “Foxp3”**

The Treg field gained momentum in 2003 with the identification of the transcription factor Foxp3 as the defining factor for this regulatory lineage. Mutations in the X-chromosome encoded Foxp3 gene were identified in human patients with IPEX disease (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) and an analogous spontaneous disease in Scurfy mutant mice, that resulted in fatal, early-onset T cell-mediated autoimmune disease manifesting as diabetes, thyroiditis, hemolytic anemia, hyper-IgE, dermatitis,

splenomegaly and lymphadenopathy and 'cytokine storm' (Bennett *et al.*, 2001; Brunkow *et al.*, 2001; Chatila *et al.*, 2000; Wildin *et al.*, 2001). This disease only affected hemizygous Foxp3 mutant males, while heterozygous mutant females were protected, owing to the presence of functional Foxp3 wild-type genes on one X-chromosome (Godfrey *et al.*, 1994). This indicated that Foxp3 mutations do not affect random X-chromosome inactivation in T cells (Fontenot *et al.*, 2005b; Gavin *et al.*, 2007). Expression of Foxp3 is specifically high in CD4<sup>+</sup>CD25<sup>+</sup> T cells and not in naïve or activated CD4<sup>+</sup> T cells. Importantly, forced expression of Foxp3 in conventional T cells converts them to Treg phenotype, exhibiting anergy and suppressive potential (Hori *et al.*, 2003). Transfer of Foxp3<sup>+</sup> Tregs to neonatal Scurfy can rescue the autoimmune disease phenotype, and the disease noted in mice with T cell specific targeted deletion of Foxp3 phenocopies the germline Foxp3 mutant mice, thus highlighting the lack of Foxp3 in T cells to be responsible for the autoimmune pathology in Scurfy mice (Fontenot *et al.*, 2003; Khattri *et al.*, 2003). Conditional deletion of Foxp3 in adult Foxp3<sup>DTR</sup> mice triggered similar fatal multiorgan immune disease, indicating that continuous expression of Foxp3 in Tregs is essential throughout the lifespan to prevent autoimmunity (Kim *et al.*, 2007; Lin *et al.*, 2007; Wan and Flavell, 2007; Williams and Rudensky, 2007).

### **Treg differentiation in the thymus and periphery**

Treg cells in the periphery have two origins – the ones that commit to the Treg fate in the thymus during development (referred as natural or 'nTregs') and

those that can be induced in the periphery from naïve CD4 T cells through signals mediated by TCR in the presence of the cytokine, TGF $\beta$  (referred as induced or 'iTregs'). At this point, there are no well-defined markers differentiating the two Treg sub-types *in vivo*. Further, it is not yet completely clear whether these two modes of Treg differentiation serve different or complementary functions *in vivo* nor is their relative contributions to the overall Treg pool under basal conditions and in response to different immune challenges well understood (Curotto de Lafaille and Lafaille, 2009).

High expression of CD25, CD5 and CTLA-4 in Tregs (molecules up-regulated with acute or chronic TCR activation) indicated that TCR signals of increased strength favor Treg cell selection during thymic differentiation (Azzam *et al.*, 2001; Carter *et al.*, 2005; Wong *et al.*, 2001). Several lines of experiments support an instructive role for TCR signaling in Foxp3 induction and Treg differentiation (Hsieh *et al.*, 2004). TCR repertoire analysis of Tregs and non-Tregs showed that Treg TCRs were of broad variety, only partially overlapping with the TCR sequences of non-Treg cells and were biased towards self-reactivity (Pacholczyk *et al.*, 2006; Pacholczyk and Kern, 2008; Wong *et al.*, 2007b). Treg cell selection is mediated by TCRs with affinities for self-peptide ranging between positive selection of conventional CD4 T cells and negative selection of high-affinity self-reactive T cells (Josefowicz *et al.*, 2012a). In addition to TCR signaling, CD28 co-stimulatory signals also play a cell-intrinsic role in Treg fate determination, as noted by marked reduction in Treg frequencies in CD28-deficient and CD80/CD86-deficient mice (Salomon *et al.*, 2000; Tai *et*

*et al.*, 2005). Several transcription factors downstream of TCR and CD28 signaling – NFAT, NF $\kappa$ B and AP-1 play a positive role in transcriptional control of Foxp3 via direct binding to the regulatory elements in the Foxp3 locus (Mantel *et al.*, 2006). The fact that the Treg cell TCR repertoire exhibited partial overlap with non-Treg cells indicated the importance of additional signals in Treg fate determination. These secondary signals were afforded by the gamma-chain signaling cytokines, primarily, IL-2 and to a lesser degree, IL-7 and IL-15 (Vang *et al.*, 2008). Mice lacking IL-2 or IL-2R $\alpha$  chain showed an almost 50% reduction in numbers of Foxp3<sup>+</sup> thymocytes (Burchill *et al.*, 2007a; Fontenot *et al.*, 2005a). Hsieh *et al.* thus proposed a two-step model for thymic Treg differentiation: Increased TCR signals up-regulate CD25 on thymic Treg precursors that increases the affinity of developing thymocytes for IL-2 and the IL-2 signals lead to subsequent Foxp3 induction and thus Treg differentiation (Burchill *et al.*, 2008; Lio and Hsieh, 2008). Indeed, the transcription factor STAT5 activated downstream of IL-2 and other gamma-chain cytokines, has binding sites in the Foxp3 promoter and Foxp3 CNS2 element and exerts positive effects on Foxp3 expression (Burchill *et al.*, 2007b; Yao *et al.*, 2007). Apart from IL-2, ablation of transforming growth factor beta receptor I (TGF $\beta$ RI) in developing thymocytes led to a transient but substantial drop in the generation of thymic Tregs during the first week of life, that was restored to levels comparable to wild-type later in development (Liu *et al.*, 2008). This suggested that similar to its role in iTreg differentiation, TGF $\beta$  signaling may be important to regulate thymic Treg differentiation through interaction with the SMAD-NFAT binding site in Foxp3-



CNS1 element (Tone *et al.*, 2008). However, recent studies have identified that TGF $\beta$  signaling in the thymus is essential to provide survival signals to thymocytes by inhibiting Bim-dependent apoptosis and is dispensable for Foxp3 up-regulation (Ouyang *et al.*, 2010).

Unlike thymic nTregs, peripheral differentiation of Tregs or iTregs likely occurs in response to non-self antigens, i.e. allergens, food or commensal microbiota, etc.. (Hsieh and Rudensky, 2005; Kretschmer *et al.*, 2005; Lathrop *et al.*, 2008). The TCR repertoire of iTregs is distinct from thymic Foxp3<sup>+</sup> Tregs and non-Tregs, as illustrated by TCRs on Tregs from the colon being different from lymph node Tregs TCRs (Lathrop *et al.*, 2011; Lathrop *et al.*, 2008). Experimental evidence points towards high-affinity TCR signaling and sub-optimal co-stimulation (increased CTLA-4 and low CD28 signaling) as optimal conditions for Foxp3 induction and iTreg differentiation (Benson *et al.*, 2007; Zheng *et al.*, 2006). In addition, TGF $\beta$ R signaling acting via Foxp3-CNS1 element is essential for most cases of peripheral induction of Foxp3 (Chen *et al.*, 2003; Zheng *et al.*, 2004). IL-2-dependent STAT5 signals also favor Foxp3 up-regulation in peripheral CD4 T cells (Davidson *et al.*, 2007; Horwitz *et al.*, 2008). In the presence of TGF $\beta$ , IL-2 inhibits differentiation to the antagonistic Th17 lineage (Laurence *et al.*, 2007). Downstream of TCR and IL-2 signaling, sustained Akt activation inhibits stable Foxp3 expression in peripheral CD4 T cells (Wong *et al.*, 2007a). This is supported by recent observations that Foxo1 and Foxo3A, which are inactivated by Akt, can directly bind to Foxp3 promoter and CNS2 element and up-regulate Foxp3 expression (Harada *et al.*, 2010; Kerdiles *et al.*, 2010).

Three main subsets of iTregs that are induced in the periphery have been described, based primarily on the cytokines that mediate their induction and function – Type 1 regulatory T (Tr1) cells are IL-10-dependent (Pot *et al.*, 2009; Vieira *et al.*, 2004), T helper 3 (Th3) cells are TGF $\beta$ -dependent (Carrier *et al.*, 2007) and iTR35 are IL-35-dependent (Collison *et al.*, 2010). Mucosal sites such as GALT and mesenteric lymph nodes have been reported as ideal environments favoring iTreg differentiation in response to chronic exposure to commensals and food derived antigens. The CD103<sup>+</sup> DCs present at these sites produce TGF $\beta$  and retinoic acid (RA) that are optimal signals for Foxp3 induction in CD4 T cells (Benson *et al.*, 2007; Coombes *et al.*, 2007; Sun *et al.*, 2007).

### **Foxp3-mediated regulation of Treg transcriptional and functional program**

The concept of a lineage-defining transcription factor is based on the premise that such a factor should be both necessary and sufficient to endow the phenotypic and functional attributes of the specific lineage. Early observations of a fatal autoimmune disease resembling the human disease IPEX in Foxp3-deficient scurfy mice and enforced Foxp3 expression able to induce suppressor potential in conventional T cells, led to the identification of Foxp3 as the ‘master’ or ‘lineage-specifying’ transcription factor for regulatory T cells (Fontenot *et al.*, 2005b; Gavin *et al.*, 2007; Hori *et al.*, 2003). A series of experiments were subsequently performed to understand the molecular underpinnings of Treg fate specification by Foxp3. Treg-specific expression of Foxp3 conferred the defining features in Tregs: anergy (lack of proliferation following TCR stimulation *in vitro*)

and suppressive capacity (Gavin *et al.*, 2007). Treg cells are unable to produce IL-2 or other gamma-chain cytokines, and they express reduced levels of IL-7R $\alpha$ . However, they up-regulate CD25 (IL-2R $\alpha$ ) in a Foxp3-dependent manner, which is essential for Treg consumption of local IL-2 made by activated T cells and thus their survival and proliferation. Treg-specific expression of Foxp3 also ensures that Treg cells do not express effector cytokines of Th1, Th2 and Th17 lineages, which otherwise could lead to dire consequences, considering the potentially self-reactive TCRs on Tregs (Lin *et al.*, 2007; Wan and Flavell, 2007). To assess the direct contributions of Foxp3 in the Treg lineage and to segregate any effects from signals that precede or promote its expression in Tregs, two different mouse models with nonfunctional Foxp3 protein expression (Foxp3<sup>gfpKO</sup> and Foxp3 $\Delta$ <sup>EGFP</sup>) were tested by different groups (Gavin *et al.*, 2007; Lin *et al.*, 2007). The resulting GFP<sup>+</sup> 'Foxp3-less' T cells retained many of the key Treg features, including the inability to produce IL-2 and an anergic phenotype, however, they lacked suppressive ability, stability and displayed reduced expression of hallmark Treg suppressor genes. These experiments indicated that Foxp3 amplifies and stabilizes features such as anergy, IL-2 dependence and expression of negative regulators of T cell activation (CTLA-4, GITR, TRAIL) induced by TCR signaling in developing Treg precursors (Josefowicz *et al.*, 2012a). Nevertheless, Foxp3 expression is indispensable for the Treg suppressor program and repression of effector cytokine gene expression. Foxp3 stabilizes Treg lineage features by regulating cell surface and signaling molecules (e.g. PDE3B), thereby maintaining Treg cell homeostasis and stability in response to different

environmental cues. Considering that part of the Treg transcriptional program is already derived from influences of TCR, IL-2 and TGF $\beta$  signaling separate from Foxp3-mediated effects, it is likely that a higher order regulation exists in the Treg lineage, upstream of Foxp3 (Hill *et al.*, 2007; Sugimoto *et al.*, 2006). Possible involvement of Ikaros family of transcription factors, specifying Treg fate upstream of Foxp3 has been proposed (Chatila, 2007). The Treg transcriptional and functional landscape relies on the high expression of Foxp3 specific to Tregs, as mice with attenuated Foxp3 protein expression (referred as 'FILIG' mice) develop fatal autoimmune disease, resembling Foxp3-deficient scurfy mice. FILIG Tregs exhibited impaired suppressor potential due to decreased Treg signature genes and secreted Th2 cytokines, resulting in Th2 differentiation of effector T cells (Wan and Flavell, 2007). In addition, Foxp3 has also been shown to stabilize and positively regulate its own expression (Bruno *et al.*, 2009; Rudra *et al.*, 2009), either via IL-2 signaling or by repressing antagonistic transcription factors like ROR $\gamma$ t (Zhou *et al.*, 2008a). Indeed continuous expression of Foxp3 in Treg cells throughout the lifespan is essential to maintain the suppressor program, as its deletion in mature Tregs results in loss of function and conversion of Tregs to pro-inflammatory cytokine producing effector T cell lineages (Williams and Rudensky, 2007). Genome wide analysis combining ChIP and tiling arrays have identified that Foxp3 directly binds to 20-30% of the Foxp3-dependent genes, and functions as both a transcriptional activator and repressor, mediating distinct regulation in the thymus and periphery (Birzele *et al.*, 2011; Marson *et al.*, 2007; Zheng *et al.*, 2007b). This dual regulation is possible due to

Foxp3-dependent specific histone modifications at binding sites in its target genes. Foxp3 target genes in the thymus primarily encode factors implicated in gene regulation and chromatin remodeling, while those shared in the periphery include Treg surface markers and intracellular signaling regulators. In addition, Foxp3 also establishes its developmental and functional program indirectly in conjunction with other transcription factors (PRDM1, CREM, IRF6, ZFPN1A2, STAT5, STAT4, STAT3, T-BET, IRF4, HIF1 $\alpha$ ). Foxp3 functioning as a homooligomer was demonstrated to interact with number of transcription factors NFAT, AML1/Runx1, HAT/HDAC complex, and NF $\kappa$ B to mediate its functions (Bettelli *et al.*, 2005; Lopes *et al.*, 2006). The Foxp3-NFAT interaction occurs via the forkhead (FKH) domain of Foxp3 and blocks NFAT-mediated transcription of IL-2, IL-4 and induces CTLA-4 and CD25 expression, thus conferring suppressor potential in activated T cells (Wu *et al.*, 2006). Interaction of AML1/Runx1 with the N-terminal region of Foxp3 between the FKH and leucine zipper is important for repression of IL-2 and suppressive activity of Tregs (Ono *et al.*, 2007). Acetylation of Foxp3 by HATs such as TIP60 increases Foxp3 binding to the IL-2 promoter, thereby facilitating optimal repression of IL-2 (Li *et al.*, 2007a). Foxp3 can also regulate the Treg transcriptional and functional program indirectly by targeting microRNAs. Indeed, Treg-specific deletion of miRNA-processing enzymes resulted in fatal autoimmunity resembling Foxp3-deficient scurfy mice and the resultant Tregs lost suppressor activity and gained effector characteristics (Liston *et al.*, 2008; Zhou *et al.*, 2008b). Foxp3 was shown to directly bind miR-155, which is critical for Treg homeostasis (Lu *et al.*, 2009a).

## **Mechanisms of Treg-mediated immune suppression**

Treg cells have emerged as key players in dominant tolerance and are implicated in curtailing inflammatory responses in a number of autoimmune diseases (Sakaguchi *et al.*, 2008). They can modulate or suppress function of a wide variety of cell populations, in diverse anatomical locations. Recent literature also demonstrates a role for Tregs in non-immunological contexts; i.e. adipose tissue Tregs control metabolic disorders, heart diseases, etc. (Ait-Oufella *et al.*, 2006; Feuerer *et al.*, 2009; Mor *et al.*, 2007). In certain cases, however, this suppressive function of Tregs can also limit beneficial protective responses against infections and anti-tumor immunity (Belkaid, 2007; Kretschmer *et al.*, 2006; Rouse *et al.*, 2006). Considerable progress has been made over the past several years in delineating the molecular mediators that contribute to the Treg arsenal. The Treg transcription factor Foxp3 confers a unique gene expression profile in Tregs that endows them with the suppressor potential. The different contact-dependent and contact-independent mechanisms used by Tregs can be grouped into four broad categories: (1) suppression by inhibitory cytokines, (2) suppression by cytotoxicity, (3) suppression by metabolic disruption and (4) suppression via modulation of dendritic cell maturation or function (Shevach, 2009; Workman *et al.*, 2009). Deletion of any one of these mechanisms does not phenocopy the disease in Scurfy mice. This suggests that either the key suppressive mechanism is yet to be identified, or that Treg suppression encompasses a multitude of molecules, with different mechanisms being operative based on the environment or context in which the Treg resides, the

target cell type that needs to be suppressed, or the specific disease and anatomical location under consideration (Vignali, 2008; Vignali, 2012; Vignali *et al.*, 2008).

Inhibitory cytokines (IL-10, TGF $\beta$  and the recently characterized IL-35) are expressed by Tregs and are the most extensively assessed cell-contact independent mode of Treg suppression (Asseman *et al.*, 1999; Bettini and Vignali, 2009; Collison *et al.*, 2007; Li *et al.*, 2007b). Each of these cytokines also function independently via a unique induced Treg population, namely Tr1 cells that function via IL-10, Th3 cells that are TGF $\beta$ -dependent and iTr35 cells that suppress via IL-35 (Carrier *et al.*, 2007; Collison *et al.*, 2010; Pot *et al.*, 2011). While the suppressive importance of both IL-10 and TGF $\beta$  in disease models of inflammatory bowel disease, allergy and asthma has been well accepted (Hawrylowicz and O'Garra, 2005; Joetham *et al.*, 2007), their importance for thymic nTreg function is not clear (Shevach, 2006). Ectopic expression of IL-35, similar to Foxp3, was capable of inducing suppressive potential in conventional CD4 T cells, suggesting that preferential Treg-specific expression of IL-35 is essential for maximal suppressive activity. Suppressive potential of IL-35 has been documented in five different disease settings using mouse models of IBD, homeostatic proliferation, Foxp3-deficient rescue, *Trichuris muris* infection and B16 melanoma (Collison *et al.*, 2010; Collison *et al.*, 2007) and IL-35 is also essential for the suppressor function of human Tregs (Chaturvedi *et al.*, 2011). While Tregs lacking either IL-10 or IL-35 exhibited defective suppressor potential, IL-10/IL-35 double deficient Tregs were fully functional *in vitro* and *in vivo*

comparable to wild-type Tregs. This was attributed to the compensatory increase in Cathepsin E (CTSE) expression on the double-deficient Tregs, that were functional via CTSE-induced release of TRAIL (member of the TNF superfamily that can mediate apoptosis, programmed necrosis or inhibit proliferation) (Pillai *et al.*, 2011). Thus, loss of a particular suppression mechanism can induce compensatory inhibitory mechanisms in Tregs.

Cytolysis by Tregs is mediated by granzyme A in humans and granzyme B in mouse Tregs (Gondek *et al.*, 2005). Granzyme B-deficient mouse Tregs exhibit reduced suppressor potential, and their cytolysis of effector T cells was perforin-independent. Tregs mediate B cell killing via granzyme B-dependent and partially perforin-dependent mechanism that leads to inhibition of B cell function. While granzyme B-dependent killing of target cells is reported in a number of Treg-dependent skin graft tolerance and tumor settings (Cao *et al.*, 2007), recent literature indicates that Tregs can also induce apoptosis in T cells via TRAIL DR5 (TNF related apoptosis inducing ligand-death receptor 5) pathway (Ren *et al.*, 2007). In addition to increased granzyme expression, galectin-1 (LGALS1), which is known to induce T cell apoptosis is also up-regulated in both mouse and human Tregs (Garin *et al.*, 2007).

Treg suppression via metabolic disruption encompasses different mechanisms. Increased CD25 (IL-2R $\alpha$ ) expression has been suggested to endow Tregs with preferential IL-2 consumption, thus functioning as an “IL-2 sink” and inducing apoptosis by depriving conventional T cells from their essential survival factor (Pandiyani *et al.*, 2007). This was supported by



observations that T cells co-cultured with Tregs die by apoptosis *in vitro*, and T cells deficient in Bim, a pro-apoptotic Bcl2-family member, are resistant to this mode of Treg suppression (Bouillet *et al.*, 1999). However, these findings have been contradicted by other reports showing that Bim-deficient, Bim/Puma-double deficient and Bcl2 transgenic T cells are all equally susceptible to Treg suppression as wild-type T cells (Szymczak-Workman *et al.*, 2011). Since IL-2 signaling contributes significantly to Treg homeostasis, it may be difficult to reconcile its separate role in Treg-mediated suppression. In addition, the ectoenzymes CD39 and CD73 expressed by Tregs were shown to induce pericellular adenosine, which can suppress effector T cells through activation of adenosine receptor 2A (Deaglio *et al.*, 2007). Adenosine binding to the receptor has also been shown to induce iTregs by inhibiting IL-6 and promoting TGF $\beta$  secretion (Zarek *et al.*, 2008). Tregs have also been shown to mediate direct suppression by the transfer of the second messenger cAMP into conventional T cells via gap junctions (Bopp *et al.*, 2007).

Treg cells can also exert indirect effects on T cells by modulating the maturation and function of dendritic cells that are essential for effector T cell activation. This is mediated via interactions between Treg surface molecules CTLA-4 and LAG-3 with CD80/CD86 and MHC class II on APCs, respectively, resulting in reduced activation of T cells by the interacting APCs (Cederbom *et al.*, 2000; Liang *et al.*, 2008; Oderup *et al.*, 2006; Read *et al.*, 2000). Indeed, intravital imaging studies have revealed direct interactions between Tregs and DCs *in vivo* (Tadokoro *et al.*, 2006; Tang *et al.*, 2006). In addition, Treg cells can

also induce production of indoleamine 2,3-dioxygenase (Akbari *et al.*) by DCs, an enzyme that can catabolize tryptophan into pro-apoptotic metabolites, that can facilitate suppression of effector T cells (Fallarino *et al.*, 2003; Mellor and Munn, 2004).

### **Phenotypic and functional specialization of Treg cells**

Since regulatory T cells are the key players in maintaining immune tolerance and homeostasis, their activities need to be finely tuned to strike the balance between restraining deleterious inflammatory responses in various autoimmune conditions, while allowing protective responses against infections and tumors. Treg cells mediate their function by curtailing the activities of diverse cell types of the innate and adaptive immune response. Considering the ever-increasing list of diverse immunological and non-immunological responses and pathologies that are under control of this suppressor T cell subset, it is not too surprising to identify ‘sub-phenotypes’ or ‘flavors’ of this regulatory lineage that are phenotypically and functionally specialized to ensure optimal regulation (Campbell and Koch, 2011; Feuerer *et al.*, 2010; Shevach, 2006). These different ‘sub-phenotypes’ are characterized by (i) differential expression of transcription factors that mediate functional specialization to control different types of immune responses, (ii) differential expression of chemokine receptors that enable trafficking of these cells to diverse lymphoid and non-lymphoid compartments, (iii) differential utilization of suppressor mechanisms to control diverse target cell

types in diverse environmental and disease contexts and (iv) different cytokine or metabolite cues that regulate their differentiation and function.

Recent studies have highlighted that Treg cells undergo functional specialization in the periphery by co-opting the transcriptional program of specific effector T cells they suppress (Figure 2). Thus, Th1-Tregs express the Th1 transcription factor T-bet and Th1-associated chemokine receptor CXCR3 and are specialized for Th1 regulation (Koch *et al.*, 2009). T-bet is the master Th1 transcription factor specifying differentiation, migration and IFN $\gamma$  production in Th1 cells. Thus, expression of T-bet endows Tregs to adopt the Th1 program and accumulate at sites of Th1 inflammatory responses (e.g. mycobacterium tuberculosis infection). T-bet-deficient Tregs fail to control Th1 responses when adoptively transferred to Foxp3-deficient scurfy mice, demonstrating the selective failure in control of Th1 inflammation. This acquisition of T-bet expression requires intact IFN $\gamma$ /STAT1 signaling in Tregs. However, unlike Th1 cells wherein the Th1 program is stabilized by IL-12/STAT4 activation of IFN $\gamma$ , this pathway is blocked in Tregs, potentially to maintain the characteristic Treg signature in T-bet<sup>+</sup>CXCR3<sup>+</sup> Tregs and prevent them from converting to Th1 effectors. Similarly, expression of the Th2 differentiation factor, IRF4, endows Tregs with the ability to control Th2 responses (Zheng *et al.*, 2009). Mice with Treg-specific deletion of IRF4 develop spontaneous Th2-mediated autoimmune lesions in pancreas, stomach and kidneys, and exhibit increased Th2 cells, spontaneous germinal centers, elevated serum IgG and IgE antibodies (Th2 isotypes) and increased plasma cells. IRF4 is also important for differentiation of Th17 and Tfh cells.

Thus, expression of IRF4 in Th2-Tregs may also be important for control of aberrant Th17 and Tfh responses as noted by increased GC activity and autoantibodies. Similar to T-bet and IRF4, Treg-specific expression of the Th17 transcription factor STAT3 is essential for control of Th17 responses (Chaudhry *et al.*, 2009). Deletion of STAT3 in Tregs provokes spontaneous intestinal inflammation with increased numbers of infiltrating Th17 cells, highlighting the selective failure in control of Th17 responses. STAT3 expression up-regulates chemokine and cytokine receptors (CCR6, IL-1R, IL-6R) in the Th17-Tregs that enable these Tregs to migrate effectively to sites of Th17 inflammation. STAT3-deficient Tregs exhibit increased expression of IL-6 and TGF $\beta$ , that can further amplify the Th17 inflammatory responses. Expression of the Tfh transcription factor Bcl6 in Tregs has recently been demonstrated to be essential for Treg-control of aberrant germinal center responses and autoantibody formation (Chung *et al.*, 2011; Linterman *et al.*, 2011). These Tfh-Tregs or Tfr cells share phenotypic characteristics with conventional Foxp3<sup>+</sup> Tregs and Tfh cells, derive from Foxp3<sup>+</sup> natural Treg precursors and are dependent on Bcl6 to adopt the Tfh cell development program. Immunization with protein antigen mediates trafficking of these cells to the germinal centers where they regulate the quality of the germinal center reaction in terms of affinity maturation of antibodies, differentiation to plasma cells and limiting outgrowth of non-antigen specific B cells. Thus, the transcription factors T-bet, IRF4, STAT3 and Bcl6 define unique functionally specialized sub-phenotypes of Tregs that each control a distinct

class of immune responses, suggesting that Tregs and effector T cells differentiate in parallel in the periphery in response to diverse inflammatory cues.

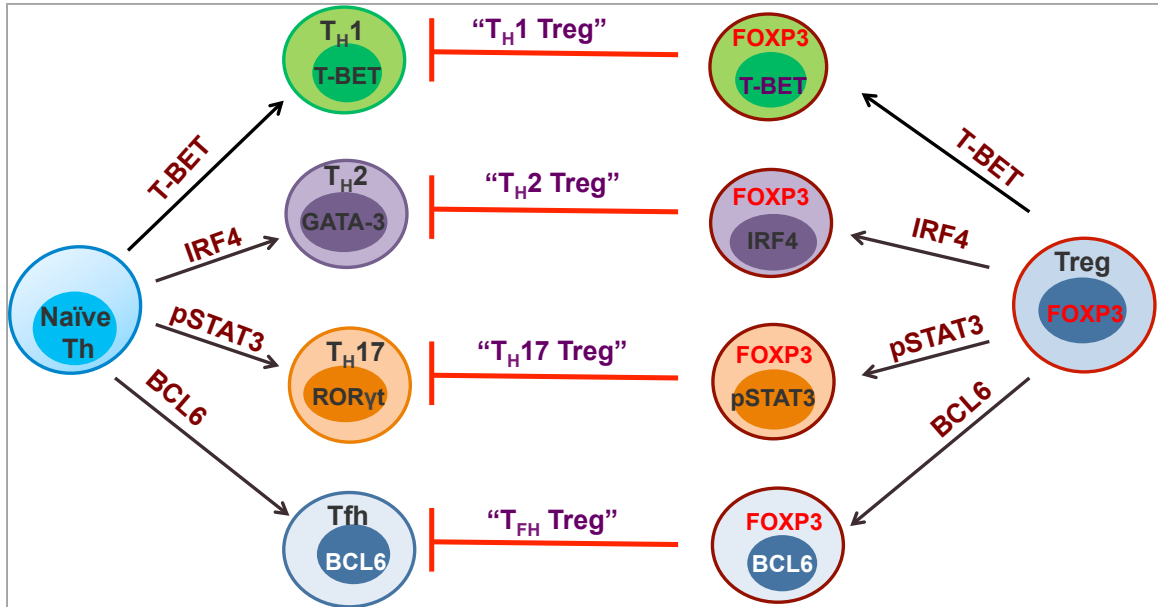
Treg cells express a diverse array of chemokine receptors and adhesion molecules that enable trafficking to lymphoid and non-lymphoid compartments, in the presence or absence of overt inflammation (Huehn *et al.*, 2004; Sather *et al.*, 2007). Expression of CD62L and CCR7 is required for Treg migration and retention in lymphoid tissues, as demonstrated by failure of CCR7-deficient Tregs to control colitis (Schneider *et al.*, 2007). Expression of P- and E- selectin ligands directs Treg homing to skin (Dudda *et al.*, 2008), CD103 to gut-associated lymphoid tissues (Suffia *et al.*, 2005), CCR9 to small intestine (Guo *et al.*, 2008), CXCR6 to liver (Lim *et al.*, 2006) and CXCR4 to bone marrow, peyer's patches and tumor sites (Wald *et al.*, 2006). In addition to guiding migration to different anatomical locations, differential chemokine receptor expression on Tregs also permits effective control of diverse immune responses – CXCR3 for control of Th1 responses (Koch *et al.*, 2009), CCR4 and CCR8 for Th2 responses (Zheng *et al.*, 2009), CCR6 for Th17 responses (Chaudhry *et al.*, 2009) and CXCR5 for control of germinal center responses (Chung *et al.*, 2011; Linterman *et al.*, 2011). Visceral adipose tissue (VAT) Tregs display a unique chemokine receptor expression pattern (elevated CCR1, CCR2, CCR9 and CXCL10 and low CXCR3) that is guided by PPAR $\gamma$ , the transcription factor that regulates accumulation, phenotype and function of this subset (Cipolletta *et al.*, 2012). A point to note is that Tregs in distinct tissues exhibit a distinct TCR repertoire, suggestive of Treg priming in response to tissue-specific antigens can guide their expression of

chemokine receptors and thus migration to different compartments to restrain immune responses.

The four major suppressive mechanisms of Tregs (release of inhibitory cytokines, cytotoxicity, metabolic disruption and DC-mediated suppression) seem to be differentially utilized by Tregs in the lymphoid and non-lymphoid compartments. The inhibitory cytokine IL-10 is particularly important for maintaining homeostasis at mucosal sites like lungs, gut and skin (Rubtsov *et al.*, 2008), but is not required for control of systemic autoimmunity. TGF $\beta$  has also been implicated in control of colitis and allergic inflammation. In addition, induced or extra-thymically generated Tregs have been demonstrated to specifically regulate inflammatory responses at mucosal sites (Josefowicz *et al.*, 2012b). On the other hand, Treg surface markers like CTLA4 and GITR function via inhibiting the immune-stimulatory activity of DCs in the lymphoid compartment (Tadokoro *et al.*, 2006). Therefore, deletion of CTLA-4 on Tregs leads to splenomegaly and lympho-adenopathy, massive multi-organ autoimmunity including myocarditis (Wing *et al.*, 2008). Similarly, deletion of GITR<sup>high</sup> Tregs induces autoimmune myocarditis and other organ-specific autoimmune diseases such as gastritis and thyroiditis (Wing *et al.*, 2008).

In addition, naïve T cells can be induced to express Foxp3 by various means: *in vitro* culture with TGF $\beta$  and IL-2, chronic stimulation with sub-optimal dose of antigen, oral delivery of antigen or homeostatic expansion (Chen *et al.*, 2003; Kretschmer *et al.*, 2005; Mucida *et al.*, 2005). The transcriptional profile of these converted Treg populations is different from the thymus-derived Tregs. In

addition, there exists considerable heterogeneity in these converted Tregs subsets generated in response to diverse cytokine and environment cues (Feuerer *et al.*, 2010). Tregs converted under 'homeostatic' conditions (following transfer of Foxp3<sup>+</sup> CD4<sup>+</sup> T cells into lymphopenic RAG-deficient mice) acquire a highly proliferative and activated KLRG1<sup>+</sup>CD103<sup>+</sup> phenotype. These Tregs preferentially express suppressor genes (CD39 and Ebi3) and transcription factors, T-bet and Eomes. Tregs recovered from the lamina propria (which are potential *in vivo* equivalents of TGFβ-converted Tregs) share many features with the homeostatically-converted Tregs (Feuerer *et al.*, 2010). Tregs generated in response to low-dose antigen (DEC-peptide converted) were relatively quiescent, exhibiting high expression of *Ctla4* and *Il10* and transcription factors, *Irf4*, *Jun* and *Fos* (Feuerer *et al.*, 2010). IL-2R signaling was recently demonstrated to induce the generation of terminally differentiated KLRG1<sup>+</sup> Treg subset, that marks highly activated antigen-responsive cells with high expression of Treg suppressor genes and which localize mainly to mucosal sites (Cheng *et al.*, 2012). The requirement for IL-2R signaling suggests that these Tregs develop at sites with strong auto-reactive T cell responses with high IL-2 levels or persistent antigen exposure such as the gut mucosa.



**Figure 2: Functional heterogeneity exhibited by Treg cells.**

Similar to naïve T cells, Treg cells also undergo functional specialization in the periphery. Thus, in addition to expressing Foxp3, Treg cells tend to co-opt the transcriptional program of the effector T cells they suppress. Whether these functionally heterogeneous Treg populations are permanently fixed or transient forms that allow effective control of the specific helper T cell response needs further investigations.



## Stability and plasticity of Treg cells

Studies with genetic tagging of Tregs have yielded compelling evidence for the stability of this regulatory T cell lineage in healthy immune settings. Foxp3<sup>+</sup> Tregs adoptively transferred into lymphopenic or Treg-deficient mice maintain high levels of Foxp3 expression, suggesting that majority of the Tregs remain stable in non-inflammatory contexts (Floess *et al.*, 2007; Gavin *et al.*, 2007; Rubtsov *et al.*, 2010). However, growing literature in the field also indicates that in inflammatory environmental settings, Treg cells can lose Foxp3 expression and/or acquire effector T cell features, suggesting that the Treg transcriptional program is not permanently imprinted (Korn *et al.*, 2007b; Tang *et al.*, 2008; Wan and Flavell, 2007; Zhou *et al.*, 2009c). Indeed, sustained expression of Foxp3 in the Treg lineage is critical for maintaining regulatory activity throughout the lifespan, as shown by the lethal autoimmune disease triggered following ablation of Foxp3 in Tregs in adult mice (Williams and Rudensky, 2007). Foxp3 expression in Tregs at any particular time derives from a combination of intrinsic cues that regulate and maintain its expression and extrinsic signals that influence Treg phenotype and function in diverse settings.

Foxp3 expression in Tregs is regulated via TCR signaling, CD28 co-stimulation and the signaling pathways activated downstream (NFAT, NFκB, AP1, CREB, ATF) (Sauer *et al.*, 2008). In addition, IL-2 and TGFβ acting via STAT5 and SMAD phosphorylation, respectively, are also important for stable Foxp3 expression in Tregs. Continued TCR stimulation and constitutive activation of PI3K/mTOR pathway down-regulates Foxp3, while treatment with

Rapamycin, an inhibitor of this pathway, augments Foxp3 expression (Haxhinasto *et al.*, 2008). The transcription factor Runx1 and its cofactor Cbfb are indispensable for optimal Foxp3 expression in Tregs. Mice harboring Runx1 or Cbfb-deficient Tregs develop autoimmunity associated with splenomegaly and lymphadenopathy, serum autoantibodies and hyper-IgE production (Egawa *et al.*, 2007; Kitoh *et al.*, 2009). This is primarily due to attenuated Foxp3 expression, reduced Treg suppressor genes and secretion of the effector cytokine, IL-4 by Runx1 or Cbfb-deficient Tregs. This demonstrates that a critical role for Foxp3 in the Treg lineage is to suppress Treg acquisition of effector features, which can lead to deleterious consequences due to the self-reactive TCRs on Treg cells. The Th2 master regulator, Gata3, expressed in Tregs in an IL-4/STAT6 independent manner, has recently been identified to be vital for optimal induction and maintenance of Foxp3 expression (Wang *et al.*, 2011; Wohlfert *et al.*, 2011). Gata3 directly binds to a regulatory region in the Foxp3 locus and promotes the activity of cis-acting elements in the Foxp3 gene, thereby exerting its positive influence on Foxp3 and Treg suppression capacity. Thus, Gata3-null Tregs exhibit reduced Foxp3 and acquire expression of Th1, Th2 and Th17 effector cytokines. Mice lacking the key microRNA-processing enzymes, Dicer and Drosha, in the Treg lineage develop fatal spontaneous multi-organ autoimmunity resembling Foxp3-deficient scurfy mice, suggesting that miRNAs are a major regulator of Foxp3 expression and Treg stability. Tregs from microRNA-deficient mice exhibit reduced suppressor function and stability and skew towards effector lineages (Liston *et al.*, 2008; Zhou *et al.*, 2008b). In this context, miRNA-10a was

recently shown to positively regulate Foxp3 expression and mark stable Treg cells, although genetic ablation of this miRNA did not affect Treg numbers or Foxp3 expression (Jeker *et al.*, 2012). Foxp3 expression in Tregs is further maintained by a positive feedback loop provided by Foxp3 itself. In addition, Foxp3 expression is also regulated by epigenetic modifications of the conserved non-coding sequences in Foxp3 locus. In particular, complete demethylation of the Treg-specific demethylated region (TSDR) corresponds to stability of Foxp3 expression, as conventional T cells or induced Tregs have a fully or partially methylated TSDR, respectively (Floess *et al.*, 2007; Huehn *et al.*, 2009). Thus, stable Foxp3 expression is further reinforced by epigenetic marks that permit the development of a stable suppressor lineage.

A number of extrinsic factors also impact stability of Foxp3 expression. Pro-inflammatory cytokines of Th1, Th2 and Th17 lineages present in diverse inflammatory settings mediate antagonistic down-regulation of Foxp3 and in some cases, conversion of Tregs to effector T cells (Pasare and Medzhitov, 2003). Transcription factors T-bet, Gata3 and ROR $\gamma$ t afford negative cross-regulation for Treg differentiation (Dardalhon *et al.*, 2008; Mantel *et al.*, 2007; Takaki *et al.*, 2008). Gata3 and STAT6 have been demonstrated to inhibit iTreg differentiation by direct binding to regulatory elements in the Foxp3 locus. The inflammatory cytokine IL-6 in conjunction with IL-1 and IL-23 induces ROR $\gamma$ t and down-regulates Foxp3 leading to concomitant production of IL-17 by the Foxp3<sup>+</sup> T cells (Lal *et al.*, 2009; Yang *et al.*, 2008a). IL-2 deficiency within inflamed pancreatic islets leads to selective Foxp3 down-regulation in the diabetic setting

(Tang *et al.*, 2008). Heterogeneity in Treg population may underlie some of the observed plasticity such that the committed Treg fraction (CD25<sup>+</sup>Foxp3<sup>+</sup>) is stable, while only the small un-committed (CD25<sup>neg</sup>Foxp3<sup>+</sup>) Treg fraction is unstable and more prone to reprogramming under inflammatory setting (Komatsu *et al.*, 2009). Also, such unstable or plastic CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs have been shown to convert to follicular helper T cells and promote the formation of germinal centers in mouse Peyer's patches (Tsuji *et al.*, 2009).

Previous studies using GFP-tagged Tregs raised the concerns that the GFP<sup>+</sup> T cells that emerged in lymphopenic settings might derive from small numbers of contaminating non-Treg cells in the Treg preparations that underwent homeostatic proliferation *in vivo* (Murai *et al.*, 2009). Recent studies therefore have employed novel dual reporter mouse models to definitively address the stability and the subsequent outcome for the Tregs that down-regulate Foxp3 in inflammatory contexts (Luche *et al.*, 2007; Srinivas *et al.*, 2001). In these models, Treg cells in a BAC Tg mice express GFP-Cre under control of the Foxp3 promoter. These mice are mated to reporter mice that express an out-of-frame LoxP-flanked YFP inserted into the Rosa26 locus, such that in Foxp3<sup>+</sup> Tregs, the activated Cre will excise the sequence flanked by the LoxP sites, thus permanently labeling these cells with YFP. Thus, even if Foxp3 expression is extinguished during the lifetime, these "exTregs" will still retain YFP expression, thus allowing for an effective strategy to distinguish Tregs that lost Foxp3 from those that never expressed it (Zhou *et al.*, 2009c). These reporter mice helped identify a sizeable fraction of exTregs (10-20%) present in the peripheral

lymphoid organs even under homeostatic conditions, and this population increases significantly under inflammatory settings (~30%). These exTregs exhibit an activated memory phenotype, with production of effector cytokines (IFN $\gamma$  and IL-17A based on the microenvironment) and are pathogenic in autoimmune disease settings. However, the notion of Treg instability has been challenged by studies using an inducible dual Foxp3 reporter mouse model, wherein GFP-Cre fusion with estrogen receptor (GFP-Cre-ERT2) is inserted into the Foxp3 locus and these mice are then mated to Rosa-YFP mice (Rubtsov *et al.*, 2010). This system allows inducible tagging of Tregs, following treatment with the ER ligand, Tamoxifen. This system demonstrated that ~96% of Tregs remain stable, when tracked over months and there is a minimal Foxp3 down-regulation in immune-deficient settings. One major difference between the two reporter systems is that while the BAC-Tg system labels Tregs right from birth, the ER system is inducible and only labels about 30% of the Tregs. Thus, while the BAC-Tg system labels all Tregs (even those cells that transiently turned on Foxp3, but didn't develop into Tregs), the ER system may only label the most stable Foxp3-expressing Tregs and fail to detect the unstable Tregs that have the potential to become exTregs. The fact that even with the ER system, a small (2-5%) of Tregs did down-regulate Foxp3 suggests that exTregs are indeed a true population of cells that down-regulate Foxp3 and can potentially be pathogenic, considering the self-reactive TCRs on Treg cells (Bailey-Bucktrout and Bluestone, 2011). They have also been demonstrated to confer a local advantage to the host by making effector T cells readily available at the site of infection. Indeed, during

*Toxoplasma gondii* infection, Treg cells reduce in numbers and the remaining Tregs make IFN $\gamma$  that helps to counteract the infection (Oldenhove *et al.*, 2009). Whether Treg instability is stochastic or reversible remains to be determined and such reversible instability may underlie the cause and protection in conditions such as chronic infections and relapsing-remitting multiple sclerosis (MS) (Kohm *et al.*, 2002). Determining whether Treg instability is the consequence or an initiator for autoimmunity is critical to faithfully apply this regulatory subset for clinical purposes.

### **Plasticity of helper T cells**

The classical view of helper T cell differentiation relies on the lineage commitment model wherein expression of a single transcription factor and concomitant cytokine profile underlies the distinct phenotype and function of each helper T cell subset. Helper T cell subsets were initially considered as terminally differentiated lineages, with irreversible commitment endpoints. This was mainly supported by experiments demonstrating that naïve T cells polarized to Th1 or Th2 fates for a certain number of cell divisions maintain their commitment even when placed into polarizing conditions favoring opposite lineages (Murphy *et al.*, 1996). This stable expression pattern was ascribed to the self-reinforcing and antagonistic regulation imposed by the lineage specific transcription factors. In addition, transmission of stable epigenetic marks at cytokine loci was proposed to further reinforce lineage commitment during subsequent rounds of cell division (Ansel *et al.*, 2003; Bird *et al.*, 1998; Grogan *et al.*, 2001). Thus, signature

cytokines were shown to bear active epigenetic marks in their designated lineages and repressive marks in the opposing lineages (Wei *et al.*, 2009). However, with the discovery of new players (Th17, Tregs, Tfh, Th9, Th22), this long-held view of helper T cells as distinct committed lineages, each defined by unique transcription factor, cytokine profile and effector function has been challenged in recent times. Accumulating studies in diverse autoimmune and inflammatory disease settings suggest that helper T cell plasticity is the norm to enable optimal protective responses in different contexts. What remains to be established yet is whether there are preferential directions for plasticity or whether multiple fates are unambiguously possible for each subset.

Flexibility in helper T cell differentiation is noted in terms of expression of transcription factors and the effector cytokines (Zhou *et al.*, 2009a). The most striking example is the observation that Gata3<sup>+</sup> Th2 cells differentiate to express Th1 genes (T-bet and IFN $\gamma$ ) in the context of viral infections (Hegazy *et al.*, 2010). Th2 cells also differentiate readily into an IL-9 secreting Th9 subset during control of helminth infections (Jager and Kuchroo, 2010). Pathogenic Th17 cells with the ability to express T-bet and IFN $\gamma$  have been identified in a number of organ-specific autoimmune disease settings, although the opposite has not yet been reported (Hirota *et al.*, 2011; Lee *et al.*, 2009). Owing to the common requirement for TGF $\beta$  during differentiation of both Th17 and Treg lineages, considerable plasticity has been noted between these lineages based on the environmental milieu (Beriou *et al.*, 2009; Bettelli *et al.*, 2006; Zhou *et al.*, 2008a). Foxp3<sup>+</sup>ROR $\gamma$ t<sup>+</sup> or Foxp3<sup>+</sup>IL-17<sup>+</sup> CD4 T cells have been observed to develop *in*

*vitro* as well as detected *in vivo* in the small intestine in both mice and humans (Voo *et al.*, 2009; Zhou *et al.*, 2008a). The propensity of Tregs to differentiate into various effector T cell lineages has been reported extensively. T-bet<sup>+</sup>Foxp3<sup>+</sup> Tregs produce IFN $\gamma$  and are critical for protective immune responses in Th1 inflammatory settings, such as Mycobacterium tuberculosis infection (Oldenhove *et al.*, 2009). The Th2 transcription factor Gata3 is expressed at high levels in Tregs at mucosal sites and plays a critical role in maintenance of Foxp3 expression during inflammatory responses (Wang *et al.*, 2011; Wohlfert *et al.*, 2011). IRF4<sup>+</sup>Foxp3<sup>+</sup> Tregs permit effective control of Th2 immune responses (Zheng *et al.*, 2009). Expression of STAT3 in Tregs is required for optimal control of Th17-associated pathology (Chaudhry *et al.*, 2009). In addition, Tregs can acquire expression of Bcl6 and differentiate to Tfh cells in the Payer's patches and contribute to production of IgA and mucosal immunity (Tsuji *et al.*, 2009). Bcl6<sup>+</sup>Foxp3<sup>+</sup> Tregs with dual characteristics of Tregs and Tfh cells play key role in the control of aberrant GC and humoral responses (Chung *et al.*, 2011; Linterman *et al.*, 2011). Tfh cells also seem to exhibit considerable plasticity such that Th1, Th2, Th17 cell lineages can be differentiated to Tfh cells while Tfh cells can also differentiate and produce cytokines of Th1, Th2 and Th17 lineages (Lu *et al.*, 2011a). The Tfh master regulator, Bcl6, is expressed in all CD4 T cells and not just Tfh cells (Lu *et al.*, 2011a; Nakayamada *et al.*, 2011). Indeed, Gata3<sup>+</sup> Th2 cells have been shown to differentiate to Gata3<sup>+</sup>Bcl6<sup>+</sup> Tfh cells during helminth infections (King and Mohrs, 2009; Zaretsky *et al.*, 2009). In addition to variability in expression of transcription factors, cytokine expression in helper T



cells is also heterogeneous. Thus, while IL-21 is considered as a Tfh cytokine, its expression is not exclusive to Tfh cells as Th1, Th2, Th9 and Th17 cells also secrete IL-21 (Kaplan *et al.*, 2011; Nakayamada *et al.*, 2011; Suto *et al.*, 2008; Wurster *et al.*, 2002). Th1, Tregs and Th9 cells can also produce IL-10, initially considered as a Th2 cytokine (Saraiva and O'Garra, 2010). These and several other examples in literature highlight the emerging concept that helper T cell differentiation relies on the coordinated expression of multiple transcription factors and the degree, timing and context of their expression guides the development of helper T cells with diverse effector and regulatory functions (Nakayamada *et al.*, 2012).

### **MicroRNA-regulation of helper T cell differentiation and plasticity**

Since their recent discovery, microRNAs (miRs) have emerged as powerful regulators of gene expression in the mammalian immune system. These short 18-22 nucleotide single-stranded RNA molecules target the 3'-UTR of their target mRNAs and mediate post-transcriptional regulation of gene expression, either by mRNA degradation or translational inhibition (Ambros, 2001; He and Hannon, 2004). More than 700 different microRNAs have been identified to date, several of these being highly conserved across species and with each one able to target multiple genes (Lodish *et al.*, 2008; O'Connell *et al.*, 2010). The cells of the immune system express nearly 100 different microRNAs that are being identified to fine-tune gene expression in a wide range of processes ranging from cell development, differentiation and function. At the

same time, dysregulated miRNA expression is commonly associated with inflammation, autoimmunity and cancer (Calin and Croce, 2006). Expression profiling of T cells have identified a plethora of microRNAs that influence T cell development in the thymus and differentiation in the periphery (Merkerova *et al.*, 2008; Monticelli *et al.*, 2005). Experiments with T cell specific deletion of the microRNA-processing enzyme, Dicer led to impaired T cell development and aberrant T cell differentiation (Cobb *et al.*, 2005; Muljo *et al.*, 2005). MiRNA-cluster 17-92 guides T cell survival and selection by targeting pro-apoptotic molecules – Bim and Pten during thymic development (Xiao *et al.*, 2008). MiR-181a fine-tunes TCR signal strength and guides T cell selection by targeting a number of phosphatases (DUSP5, DUSP6, SHP2 and PTPN22) (Li *et al.*, 2007c). T cell responses in Dicer-deficient mice are skewed towards a Th1 phenotype, demonstrating microRNA-mediated control of T cell differentiation (Cobb *et al.*, 2005; Muljo *et al.*, 2005). MiR-155 targets the Th2 transcription factor, *maf* and directs towards Th1 fate (Rodriguez *et al.*, 2007; Thai *et al.*, 2007), while miR-326 drives Th17 differentiation by targeting a repressor of Th17 cells, *Ets1* (Du *et al.*, 2009). MiR-29 family members inhibit Th1 differentiation by targeting the main Th1 transcription factors, T-bet and Eomes (Steiner *et al.*, 2011). The microRNA-mediated repression pathway is likely crucial for the development of T follicular helper cells, with miR-17-92 cluster targeting the follicle-homing chemokine receptor, CXCR5 and miR-101-103 targeting the co-stimulatory molecule, ICOS (Yu *et al.*, 2009). MiR-21 regulates the Th1 vs. Th2 balance by targeting IL-12 $\alpha$ , the Th1 polarizing myeloid cell cytokine and thus

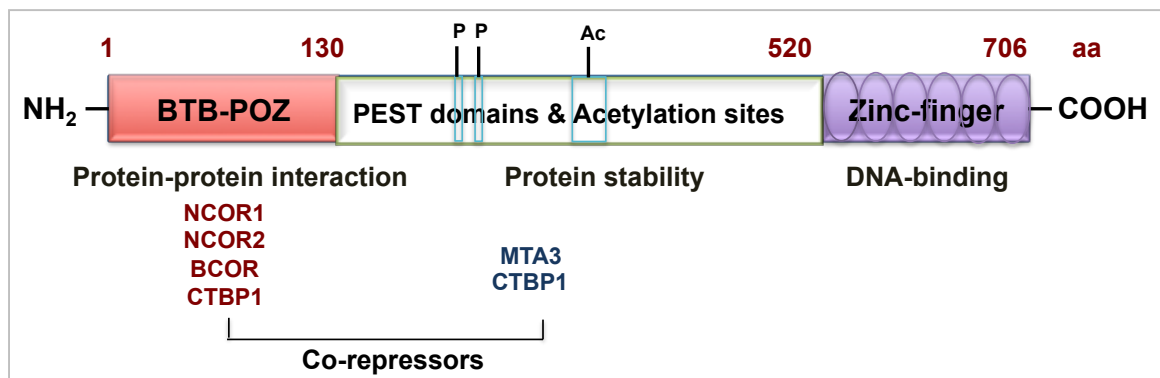
indirectly promotes Th2 differentiation (Lu *et al.*, 2011b; Lu *et al.*, 2009b). Dicer or Drosha-deletion in the Treg lineage resembles the spontaneous inflammatory disease in Foxp3-deficient mice, highlighting the importance of miRNAs in Treg stability and suppressor function (Chong *et al.*, 2008; Liston *et al.*, 2008; Zhou *et al.*, 2008b). The spontaneous disease in mice with Treg-specific deletion is triggered early in life, while the autoimmunity with T cell-specific deletion sets in later as the mice age. The Treg miRNA signature is Foxp3-dependent, and at the same time, miRs have been identified that regulate Foxp3 expression, suggesting a positive feedback loop between Foxp3 and miRNAs. A number of miRs are preferentially expressed in Tregs (miR-21, miR-223, miR-146, miR-22, miR-23a and b, miR-24, miR-214, miR-155) while several others are down-regulated (miR-142-5p and 3p, miR-30 b, c, e and Let-7 family members) (Cobb *et al.*, 2006). Subsequent studies have unraveled that these miRNAs regulate different facets of Treg biology. MiR-155 is important for Treg survival and homeostasis by targeting Socs1 and thus regulates their responsiveness to IL-2 (Lu *et al.*, 2009a). MiR-146a is critical for Treg control of Th1 inflammatory responses by targeting Stat1 (Lu *et al.*, 2010). In the absence of this miRNA in Tregs, Tregs exhibit increased Stat1 phosphorylation, acquire a Th1-bias and fail to control Th1 responses. MiR-142-3p represses cAMP production, which is important for Treg suppressor function (Huang *et al.*, 2009). MiR-10a has recently been demonstrated as a marker of stable Tregs, although the exact target genes were not identified (Jeker *et al.*, 2012). MiR-21 can indirectly positively regulate Foxp3 expression in human Tregs, while miR-31 represses

Foxp3 expression (Rouas *et al.*, 2009). Understanding the functional significance of the other Treg and T cell-specific miRNAs will advance our knowledge about the different mediators governing the biology of these cells and lead to development of more refined therapies for control of diverse pathologies.

### **Regulation of gene expression by the transcription factor Bcl6**

The Bcl6 gene encodes a 706 amino acid (95 kDa) nuclear phosphoprotein that functions as a sequence-specific transcriptional repressor (Chang *et al.*, 1996). This gene was originally cloned due to its involvement in chromosomal translocations affecting band 3q27 in non-Hodgkin's lymphoma (NHL) (Baron *et al.*, 1993; Kerckaert *et al.*, 1993; Miki *et al.*, 1994; Ye *et al.*, 1993a; Ye *et al.*, 1993b). Bcl6 (also known as LAZ3 and ZBTB27) is a member of the POK/ZBTB family of transcription factors with critical roles in development, differentiation and lymphoma-genesis (Lee and Maeda, 2012). Similar to other POK/ZBTB family members (including LRF, BAZF, MIZ1, PLZF), the structure of Bcl6 protein comprises of 6 Kruppel-type C-terminal zinc-finger motifs and an N-terminal POZ motif (also called BTB or ZIN) (Figure 3). The C-terminal zinc-fingers are important for specific DNA binding (Kawamata *et al.*, 1994), while the 120 amino acid BTB/POZ domain allows for homo-dimerization or hetero-dimerization with other proteins (Bardwell and Treisman, 1994; Okabe *et al.*, 1998). The POZ domain is mainly responsible for the repressor function of Bcl6 due to its ability to recruit co-repressors such as N-CoR (nuclear co-repressor), SMRT/NcoR2 (silencing mediator of retinoic acid and thyroid hormone receptor),

BCOR (Bcl6 interacting corepressor), and HDACs (histone deactelyases) to promoter regions of target genes (Chang *et al.*, 1996; Dhordain *et al.*, 1997; Huynh and Bardwell, 1998; Huynh *et al.*, 2000; Wong and Privalsky, 1998). The POZ domain also allows interactions of Bcl6 with other POK family members, particularly BAZF, MIZ-1 and PLZF (Lee and Maeda, 2012). The intervening central region between the POZ domain and the zinc-finger, which includes 3 PEST motifs, has also been shown to mediate repression independently (Albagli *et al.*, 1995; Parekh *et al.*, 2007; Seyfert *et al.*, 1996). Thus, the function of Bcl6 in a particular cellular context is dependent on the availability of different co-repressors or interacting partners in the nuclear milieu (Crotty, 2011; Parekh *et al.*, 2008). The DNA-binding motif of Bcl6 shares close resemblance to the “GAS” motif recognized by the cytokine-induced STAT-family transcription factors (Dent *et al.*, 1997; Seyfert *et al.*, 1996).



**Figure 3: Schematic representation of Bcl6 protein.**

The 3 main domains of Bcl6 protein are displayed: BTB/POZ domain at the N-terminal that mediates interactions with co-repressors and other BTB family members, central PEST domain with the phosphorylation (P) and Acetylation (Ac) sites that confers protein stability and also recruits co-repressors and the 6 C-terminal Zn fingers that are critical for DNA binding.

## **Bcl6 as the master regulator of germinal center (GC) B cells**

Rearrangements of the Bcl6 gene have been associated with 30-40% of diffuse large cell lymphoma and 6-11% follicular lymphoma cases (Bastard *et al.*, 1994; Lo Coco *et al.*, 1994; Otsuki *et al.*, 1995). Most of these rearrangements involve translocation of heterologous promoters derived from other chromosomes to Bcl6 coding region leading to deregulated Bcl6 expression (Ye *et al.*, 1995). Point mutations in the 5' non-coding region of Bcl6 have also been linked to lymphomagenesis (Dalla-Favera *et al.*, 1994; Migliazza *et al.*, 1995). Bcl6-associated lymphomas are frequently derived from germinal center (GC) B cells. Within the B cell lineage, the expression of Bcl6 is specifically confined to GC B cells and almost undetectable in pre-GC cells and terminally differentiated plasma cells (Cattoretti *et al.*, 1995; Flenghi *et al.*, 1995; Onizuka *et al.*, 1995). The GC stage of B cell differentiation favors maturation of the antibody response, qualitatively and quantitatively with processes such as somatic hypermutation, affinity maturation, isotype-switching and memory B cell formation (MacLennan, 1994). Bcl6 acts intrinsically in B cells to prevent the terminal differentiation of GC B cells to plasma cells or memory cells, via repression of plasma cell differentiation factor, BLIMP1 (Prdm1) (Reljic *et al.*, 2000; Shaffer *et al.*, 2000). Thus, mice deficient in Bcl6 lack GCs and fail to mount protective antibody responses (Dent *et al.*, 1997; Fukuda *et al.*, 1997; Ye *et al.*, 1997). Constitutive expression of Bcl6 resulting from point mutations or translocations such as those in lymphomas, maintains B cells in the actively proliferating and mutating GC

phenotype leading to B cell transformation (Pasqualucci *et al.*, 2003; Wang *et al.*, 2002).

Considering the oncogenic role of Bcl6 in the B cell lineage, multiple target genes for Bcl6 have been characterized in this lineage. One of the key target gene is the plasma cell differentiation factor, BLIMP1 (Reljic *et al.*, 2000; Shaffer *et al.*, 2000). In addition, Bcl6 inhibits cell-cycle regulators (cyclin D2, p21<sup>cip1</sup>, p27<sup>kip1</sup>) and checkpoint controls (ATR, p53) that correspond with its role in promoting GC B cell proliferation and B cell transformation (Baron *et al.*, 2002; Phan and Dalla-Favera, 2004; Phan *et al.*, 2005; Shaffer *et al.*, 2000). More recently, gene expression profiling in combination with ChIP-on-chip approaches have identified over 1200 genes whose promoters are bound by Bcl6 and down-regulated in GC B cells (Basso *et al.*, 2010; Ci *et al.*, 2009). While a number of these gene targets confirm the previously recognized role for Bcl6 in B cell activation, differentiation and survival, these approaches have uncovered novel roles for Bcl6 in regulating signaling via Toll-like receptor, IFN-R, TGF-R, cytokine and Wnt pathways (Basso *et al.*, 2010). Thus, the multitude of target genes and the cellular pathways they regulate suggest that the transcriptional modulation by Bcl6 in GC B cells orchestrates controlled expression of molecules critical for the GC reaction (Basso and Dalla-Favera, 2010).

## **Bcl6 as an arbiter of helper T cell differentiation program**

### **Bcl6 functions in Th1 cells**

Bcl6 expression is higher in Th1 cells, relative to Th2 cells, consistent with the role for Bcl6 in inhibiting Th2 gene expression program in Th1 cells (Cimmino *et al.*, 2008; Mondal *et al.*, 2010). The mechanisms for repression of Th2 differentiation by Bcl6 include competition with STAT6 for binding to Th2 genes (Dent *et al.*, 1997; Harris *et al.*, 1999; Harris *et al.*, 2005), binding to a silencer in the Il5 gene (Arima *et al.*, 2002) and post-transcriptional control of the Th2 transcription factor Gata3 (Kusam *et al.*, 2003). Analogous to the regulation of B cell differentiation program, antagonistic regulation of the transcription factor BLIMP1 by Bcl6 in T cells is critical for the Th1 vs. Th2 development (Cimmino *et al.*, 2008). BLIMP1 is highly expressed in Th2 cells and is critical for Th2 humoral responses *in vivo*, but is dispensable for Th2 differentiation *in vitro*. BLIMP1 directly binds and represses Th1 genes, IFN $\gamma$  and Tbx21 and thus polarizes towards Th2 fate (Cimmino *et al.*, 2008). Thus, the repression of BLIMP1 by Bcl6 in Th1 cells is critical to block the ability of BLIMP1 to inhibit Th1 gene expression. Yu *et al* reported increased percentages of IFN $\gamma$ -producing cells within the Bcl6-deficient T cell compartment in SRBC-immunized Bcl6<sup>+/+</sup>: Bcl6<sup>-/-</sup> fetal-liver chimeras, suggesting that Bcl6 was not required for Th1 cell development (Yu *et al.*, 2009). Bcl6 represses Th1 differentiation, via direct binding to the T-bet gene promoter (Yu *et al.*, 2009). Retrovirus-mediated over-expression of Bcl6 suppressed Th1 differentiation in a dose-dependent manner (Yu *et al.*, 2009). This effect is consistent with Tfh cells (which express high



levels of Bcl6) isolated *ex vivo* and differentiated *in vitro* displaying low levels of Th1 genes, T-bet and IFN $\gamma$  (Lu *et al.*, 2011a). Similar results with ectopic Bcl6 expression inhibiting T-bet and IFN $\gamma$  gene expression were reported by Nurieva *et al.*, who further demonstrated that this repression was dependent on the DNA-binding ability of Bcl6 zinc-fingers (ZF3 and ZF5) (Nurieva *et al.*, 2009). The Th1 transcription factor T-bet was recently shown to physically associate with Bcl6 at its C-terminal zinc-finger domain and directly target promoters of genes involved in inhibiting Th1 differentiation, *Socs1*, *Socs3* and *Tcf7* (Oestreich *et al.*, 2011). In addition, Bcl6 itself gets recruited at the IFN $\gamma$  locus in a T-bet-dependent manner during later time points in Th1 differentiation and directly inhibits IFN $\gamma$  transcription. Thus, the coordinated regulation of the Th1 gene program by T-bet and Bcl6 ensures maintenance of optimal levels of IFN $\gamma$  essential for effective pathogen clearance. Forced expression of Bcl6 even in fully committed Th1 cells inhibits Th1 differentiation indicating that Bcl6 can polarize committed Th1 cells towards Tfh fate (Lu *et al.*, 2011a). A recent study also provides ample evidence for the opposite scenario: differentiation of Tfh cells to Th1 effectors (Lu *et al.*, 2011a). In fact, priming of naïve T cells with the Th1-polarizing cytokine IL-12 induces generation of IFN $\gamma$ - and IL-21- secreting cells, which resemble both Th1 and Tfh cells (Nakayamada *et al.*, 2011). The Bcl6 gene locus is accessible in naïve CD4 T cells as well as fully differentiated Th1 cells (Lu *et al.*, 2011a). Bcl6 is induced following T cell stimulation and further following cytokine-induced activation of STAT4 and STAT3 (Nakayamada *et al.*, 2011). Repression of Bcl6 by T-bet during later time points is critical for full Th1 differentiation. Thus,

antagonistic regulation of T-bet and Bcl6 determines the eventual outcome for a developing helper T cell; with chronic antigenic stimulation favoring sustained Bcl6 expression and thus Tfh differentiation while conditions favoring sustained T-bet expression have reduced Tfh differentiation. The Tfh differentiation potential of Th1 cells is further subject to regulation by IL-2 induced STAT5 signaling inhibiting Bcl6 expression in Th1 cells (Oestreich *et al.*, 2012). Th1 cells maintained in low IL-2 conditions were subject to Bcl6 up-regulation by Foxo transcription factors, leading to repression of BLIMP1 and up-regulation of Tfh genes.

### **Bcl6 functions in Th2 cells**

Earlier insights about the role of Bcl6 in the T cell lineage mainly came from studying the phenotype of Bcl6-deficient mice that develop a spontaneous Th2-mediated inflammatory disease in their heart and lungs (Dent *et al.*, 1997; Ye *et al.*, 1997). The inflammation is predominantly Th2 biased, characterized by infiltration of monocytes, macrophages, eosinophils and Th2 cells. Increased numbers of IgE-positive B cells and mast cells have also been noted in Bcl6-deficient mice. Bcl6-deficient mice also exhibit pronounced Th2 responses when immunized with a protein antigen in adjuvant, suggesting that the Th2 response can be antigen-dependent (Dent *et al.*, 1998a). These initial studies characterized Bcl6 as a critical transcription factor for inhibiting Th2 differentiation in T cells. Mice doubly-deficient in Bcl6 and STAT6 or Bcl6 and IL-4 however developed similar Th2 inflammation in heart and lungs as Bcl6-deficient mice,

indicating that Bcl6 regulates Th2 gene expression program via an IL-4/STAT6-independent pathway (Dent *et al.*, 1998a). Post-transcriptional regulation of the master Th2 transcription factor Gata3 by Bcl6 explains the STAT6-independent Th2 gene expression of Bcl6-deficient T cells (Kusam *et al.*, 2003). Transgenic mice overexpressing Bcl6 in CD4 T cells (Ick-Bcl6 Tg strain) exhibit reduced Th2 cytokines and eosinophil accumulation in the bronchoalveolar lavage (BAL) after aeroallergen challenge (Arima *et al.*, 2002). Variants of Bcl6 gene have been associated with atopy characterized by high IgE titers and positive RAST scores to mixtures of house-dust mite and grass pollen in a large-scale study with British and Japanese populations (Adra *et al.*, 1998). STAT factors and Bcl6 share common recognition motifs and Bcl6 can compete with STAT6 for repressing IL-4 transcription (Harris *et al.*, 1999; Harris *et al.*, 2005). Bcl6 also has been shown to bind to a silencer element in the IL-5 gene (Arima *et al.*, 2002). Bcl6 functions in the non-lymphoid compartment are also important in the regulation of Th2 differentiation. Bcl6 was noted to repress chemokine gene expression (MCP-1, MRP-1, MCP-3) in macrophages that can contribute to increased Th2 differentiation seen in Bcl6-deficient mice (Toney *et al.*, 2000), particularly, since MCP-1 can itself prime Th2 responses indirectly by inhibiting IL-12 from monocytes and macrophages. In addition, Bcl6 also represses IL-6 transcription in macrophages, thus further inhibiting the promotion of hyper-Th2 responses by Bcl6-deficient APCs (Yu *et al.*, 2005). Bcl6-deficient bone marrow mast cells (BMMCs) also produced increased Th2 cytokines following activation than wild-type counterparts, indicating a role for Bcl6 in inhibiting Th2 genes in mast cells

(Ohtsuka *et al.*, 2005). Bcl6-deficient dendritic cells (DCs) produce more IL-6 and less IL-12 than wild-type DCs, suggesting that repression of IL-6 by Bcl6 was also important to limit Th2 skewing (Ohtsuka *et al.*, 2011). Forced expression of Bcl6 in developing Th2 cells significantly inhibited Th2 genes (Gata3 and IL-4) (Kusam *et al.*, 2003) and this effect was dependent on Bcl6 zinc fingers (ZF3 and ZF5) (Nurieva *et al.*, 2009). Similar results were obtained by Yu *et al.* who reported almost 60% reduction of Gata3 protein following overexpression of Bcl6 in Th2 polarized cells (Yu *et al.*, 2009). These effects are concomitant with reduced expression of Th2 genes by Bcl6-expressing Tfh cells (Lu *et al.*, 2011a). Positive epigenetic marks on Gata3 in Tfh cells and on Bcl6 in non-Tfh cells suggest potential for developmental plasticity between these lineages (Lu *et al.*, 2011a). Indeed, *ex vivo* isolated Tfh cells produced Th2 effector cytokines following repolarization *in vitro*, suggesting that Tfh cells could convert to Th2 effectors. At the same time, Th2 effectors can be reprogrammed to Tfh fate following either forced expression of Bcl6 or differentiation under Tfh conditions. *In vivo*, Tfh cells have also been shown to differentiate from Th2 effectors following immunization with helminth antigens (King and Mohrs, 2009; Zaretsky *et al.*, 2009).

## **Bcl6 functions in Th17 cells**

Considerable variations in the Th2 disease severity, and the pro-inflammatory nature of Th17 cells, prompted investigations into the contributions of Th17 cells to the inflammatory disease observed in Bcl6-deficient mice. Indeed, IL-17 levels in the heart and lungs segregated with disease severity similar to IL-4, suggesting that IL-17 and thus Th17 cells were associated with disease exacerbations in Bcl6-deficient mice (Mondal *et al.*, 2010). Bcl6-deficient mice had increased IL-4 and IL-17 producing memory T cells, demonstrating that in addition to Th2, Bcl6 is also an inhibitor of Th17 responses. Highest Bcl6 levels (both transcript and protein) were noted in conditions favoring Th17 differentiation relative to Th1 or Th2 conditions, implying functional role for Bcl6 in the Th17 lineage. Bcl6-deficient naïve T cells demonstrated reduced Th17 differentiated potential *in vitro* compared to wild-type T cells, owing to their preferred Th2 bias, which could be restored by either increasing the dose of TGF $\beta$  in culture, using sort-purified naïve CD4<sup>+</sup> T cells (CD62L<sup>+</sup>CD44<sup>low</sup>) or Bcl6/IL-4 double-deficient T cells for differentiation. This demonstrated that the critical role for Bcl6 in Th17 differentiation is to inhibit IL-6 driven Th2 differentiation that can negatively impact Th17 polarization. Ectopic expression of Bcl6 in T cells promoted a modest up-regulation of IL17, with no effect on ROR $\gamma$ t, which could be attributed to its inhibitory effect on Th2 differentiation. Bcl6-deficient macrophages produced increased levels of pro-Th17 cytokines (IL-6, TGF $\beta$  and IL-23) following LPS stimulation than wild-type macrophages and conditioned media from Bcl6-deficient macrophages augmented Th17

differentiation of T cells *in vitro*. Thus, the increased Th17 responses of Bcl6-deficient T cells *in vivo* can be attributed to the pro-Th17 cytokine production of Bcl6-deficient macrophages. Thus the increased Bcl6 in Th17 conditions is critical to limit Th2 skewing and allow for optimal Th17 differentiation. While Bcl6 overexpression modestly promotes IL-17, the role of Bcl6 in repressing pro-Th17 cytokines in APCs seems more dominant *in vivo*. This result conflicts with work from Yu *et al* and Nurieva *et al* who demonstrated repression of IL-17 following Bcl6 overexpression. Nurieva *et al* demonstrated direct binding of Bcl6 to ROR $\gamma$ t gene promoter contributing to the repression of IL-17 (Nurieva *et al.*, 2009). While Yu *et al* attributed the down-regulation of IL-17 to inhibition of transcriptional activity of ROR $\gamma$ t by Bcl6, but not its expression (Yu *et al.*, 2009). Possible explanation for the observed discrepancy between these studies and the results from Mondal *et al* (Mondal *et al.*, 2010) could be explained by the differences in duration of culture with Bcl6 retrovirus, with long-term cultures potentially favoring an outgrowth of Tfh cells. Th17 genes (ROR $\gamma$ t and IL-17) are expressed at low levels in Bcl6-expressing Tfh cells (Lu *et al.*, 2011a). Positive chromatin marks on Rorc in Tfh cells and Bcl6 in non-Tfh cells highlight the plasticity shared by these lineages. Indeed, Th17 cells can be reprogrammed towards the Tfh lineage by either forced expression of Bcl6 or differentiation under Tfh conditions. While Tfh cells isolated *ex vivo* can produce Th17 effector cytokines following repolarization *in vitro*. Finally, IL-17 producing Tfh cells (referred as “Tfh17” cells) have been shown to induce ectopic follicles in the

lungs of neonates following LPS-triggered pulmonary inflammation (Rangel-Moreno *et al.*, 2011).

### **Bcl6 functions in Tfh cells**

Bcl6 has emerged as the master transcription factor specifying the T follicular helper (Tfh) cell lineage, helper T cells that provide selection signals to B cells, which is essential for generation of long-lived antibody responses (Johnston *et al.*, 2009; Nurieva *et al.*, 2009; Yu *et al.*, 2009). Ectopic expression of Bcl6 promoted expression of hallmark Tfh molecules (CXCR5, CXCR4, PD-1, ICOS, IL-21R) in T cells, thereby skewing towards Tfh fate. Bcl6-deficient T cells fail to differentiate into Tfh cells *in vivo*, and mice deficient in Bcl6 lack germinal centers and fail to mount protective antibody responses. While Bcl6 is required for both GC B cells and Tfh cells, it controls Tfh differentiation program by regulating completely different set of genes than GC B cells (Crotty, 2011). While the three initial studies using elegant gain-of-function and loss-of-function approaches firmly established the cell-autonomous role for Bcl6 in Tfh fate determination, each provided unique mechanistic insight into how a transcriptional repressor can positively direct Tfh differentiation (Bi and Ye, 2010). Johnston *et al* (Johnston *et al.*, 2009) identified the reciprocal relationship between Bcl6 and BLIMP1 in Tfh cells and reported repression of the antagonistic Tfh transcription factor BLIMP1 by Bcl6 as essential to drive differentiation from effector towards Tfh lineage. Both Nurieva *et al* (Nurieva *et al.*, 2009) and Yu *et al* (Yu *et al.*, 2009) demonstrated that Bcl6 directs Tfh

differentiation by inhibiting differentiation to alternative non-Tfh lineages. Bcl6 was shown to directly bind the promoters of T-bet and Rorc genes, or repress the transcriptional activity of Rorc. Ectopic expression of Bcl6 significantly suppressed Th1, Th2 and Th17 gene expression in a dose-dependent manner, and this effect was dependent on the DNA-binding ability of Bcl6 (Nurieva *et al.*, 2009). Yu *et al* reported an interesting aspect of Bcl6-mediated gene regulation in T cells, i.e. ability of Bcl6 to repress more than half of the abundantly expressed microRNAs in T cells by 50% or more (Yu *et al.*, 2009). Repression of microRNAs that target Tfh molecules (e.g., miR-17-92 that targets CXCR5 and miR-101-103 that targets ICOS) was revealed as a novel mechanism by which Bcl6 positively induced Tfh differentiation. Bcl6 alone does not induce the entire Tfh signature in case of human CD4 T cells; the transcription factor cMAF induces IL-21 and thus collaborates with Bcl6 for optimal Tfh differentiation (Kroenke *et al.*, 2012). Differentiation of naïve T cells with IL-6 and IL-21 was shown to up-regulate Bcl6 expression, although both the cytokines are reported to be dispensable for Tfh differentiation *in vivo* (Poholek *et al.*, 2010). The signals regulating expression of Bcl6 in CD4 T cells remain relatively unknown. ICOS has been shown to induce Bcl6 expression and thus set an early bifurcation point for Tfh vs. effector T cell differentiation (Choi *et al.*, 2011). Studies using a Bcl6 reporter mice to track Bcl6 protein expression *in vivo* have shown that Bcl6 protein gets induced in antigen-engaged CD4 T cells prior to antigen-engaged B cells, and its expression in CD4 T cells is further induced following cognate B cell interactions (Okada *et al.*, 2012). Bcl6 protein however gets gradually lost on Tfh



cells, while its maintained on GC B cells. Whether these BCL6<sup>lo</sup> Tfh cells re-differentiate towards other effector lineages, stay in GCs as memory Tfh cells or become a part of the circulating CD4<sup>+</sup>CXCR5<sup>+</sup> memory T cell pool warrants additional studies. The presence of active chromatin marks on Bcl6 in non-Tfh cells and on T-bet, Gata3 and Rorc in Tfh cells highlights the potential of plasticity between these different helper T cell lineages (Lu *et al.*, 2011a). Indeed, ectopic expression of Bcl6 or differentiation under Tfh conditions can drive Th1, Th2 and Th17 cells towards Tfh fate, while *ex vivo* isolated Tfh cells can re-differentiate towards effector lineages under appropriate conditions.

### **Bcl6 functions in Treg cells**

The severe Th2 inflammatory heart and lung pathology that develops spontaneously at an early age in majority of the Bcl6-deficient mice underscores the role for Bcl6 in regulatory T cell lineages. However, in comparison to the in-depth assessment of Bcl6 functions in effector T cell subsets, not much is known about the role of Bcl6 in conventional CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells. No significant difference in Foxp3<sup>+</sup> Treg cell percentages derived from Bcl6-deficient and Bcl6-sufficient cells were noted in fetal liver chimeras, suggesting that Bcl6 is dispensable for Treg cell development (Yu *et al.*, 2009). Chung *et al* (Chung *et al.*, 2011) however reported increased numbers of Foxp3<sup>+</sup> T cells in the thymus and spleen, but not mesenteric lymph nodes of Bcl6-deficient mice, relative to wild-type mice. Bcl6-deficient T cells exhibit a reduced tendency to differentiate into induced Treg (iTreg) lineage following *in vitro* culture with TGFβ, owing to

their intrinsic Th2 bias, which could be restored by treatment with anti-IL-4 (Nurieva *et al.*, 2009). CD25<sup>hi</sup>CD4<sup>+</sup> Treg cells from Bcl6-deficient mice express increased levels of effector lineage transcription factors (*Gata3*, *Irf4*, *Rorc*), increased *Blimp1* and Treg cytokine *Il10*, and reduced Tfh markers (*Cxcr5*, *Pd1*, *Btla*, *Pdcd1*) and these cells maintain intact suppressor function *in vitro* (Chung *et al.*, 2011). However, these studies by Chung *et al* use CD25<sup>hi</sup>CD4<sup>+</sup> Treg cells, a fraction which contains high numbers of Th2 effectors that are Foxp3<sup>-</sup> and thus not pure Tregs. Hence a detailed assessment of phenotype and *in vivo* functional potency of pure CD25<sup>+</sup>Foxp3<sup>+</sup> Bcl6-deficient Tregs is still lacking. Recent studies have focused on a specific subset of Bcl6-expressing Treg cells that are critical to regulate the germinal center reaction, referred as T follicular regulatory cells (Tfr cells) (Chung *et al.*, 2011; Linterman *et al.*, 2011). Bcl6-deficient mice lack CXCR5<sup>+</sup>Foxp3<sup>+</sup> Tfr cells. Tcr $\beta$ -deficient mice receiving CD25<sup>hi</sup>CD4<sup>+</sup> Bcl6-deficient Tregs with naïve CD4<sup>+</sup> T cells and immunized with NP-KLH in CFA displayed increased numbers of GL7<sup>+</sup>CD95<sup>+</sup> germinal center B cells, increased titers of NP-specific global and high affinity IgG isotypes as well as increased numbers of NP-specific B220<sup>+</sup> B cells and B220<sup>-</sup>CD138<sup>+</sup> plasma cells, demonstrating that Bcl6-deficient Tregs have substantial defects in control of germinal center B cells, antibody affinity maturation and plasma cell differentiation (Chung *et al.*, 2011). No obvious differences in Th1, Th2 and Th17 responses were noted in this system for mice receiving Bcl6-deficient vs wild-type Tregs (Chung *et al.*, 2011). However, here again, the Treg cells used were not selected for Foxp3<sup>+</sup> Tregs and the experimental design didn't include

appropriate control mice receiving no Tregs. Thus, a comprehensive assessment of the role of Bcl6 in these different regulatory lineages and their implications in control of T and B cell tolerance is warranted.

## RESEARCH AIMS

The overall aim of this research centered on understanding the role of the transcription factor Bcl6 in Treg cell biology and determining how that relates to the inflammatory pathology that develops in the Bcl6-deficient mice. The inflammatory disease is mainly Th2-biased, and manifests as myocarditis and pulmonary vasculitis by 4-6 weeks of age. The disease is spontaneous, T cell-dependent and resembles the pathology noted in mice with Treg-cell defects. Considering the central importance of Treg cells in limiting T cell proliferation and maintenance of immune homeostasis, the overall project focused on a detailed characterization of the phenotype and function of Bcl6-deficient Tregs to understand the molecular underpinnings underlying the control of inflammation by Bcl6.

Bcl6 has gained considerable attention in recent years due to its identification as a master transcription factor specifying the T follicular helper cell lineage. Being a well-characterized transcriptional repressor, Bcl6 has been demonstrated to positively direct Tfh differentiation indirectly by repressing microRNAs that target Tfh genes and by repressing differentiation towards non-Tfh fates by inhibiting Blimp1. In addition to directing Tfh differentiation, Bcl6 has also been shown to be indispensable for the differentiation of a specific Treg subset named T follicular regulatory cells that regulate the germinal center response.

Bcl6 has previously been reported to repress Th2 differentiation and thus Th2 inflammation by inhibiting the Th2 transcription factor Gata3 in T cells. Apart

from being the key Th2 transcription factor, Gata3 has been identified to be essential for optimal Treg functional fitness. Since wild-type Tregs express Gata3, understanding the factors that regulate Gata3 expression in Tregs and prevent the transactivation of Th2 cytokines in Tregs has been an important question in the Treg field. The first aim therefore was to determine whether Bcl6 regulation of Gata3 also operates in the Treg lineage and understanding how this regulation is crucial for Treg control of inflammatory Th2 responses.

Repression of microRNAs has emerged as an important facet of Bcl6-mediated gene regulation in T cells from studies of its role in Tfh cells. MiRNAs have been identified as critical players in T cell differentiation pathways and particularly in maintaining Treg lineage stability in inflammatory contexts. Hence, the second aim was to assess whether Bcl6 regulated specific miRNAs in the Treg lineage that can impact the Bcl6-deficient Treg phenotype and thereby affect Treg control of Th2 immune responses. MiRNA signatures are being developed as diagnostic markers for cancer detection. Hence, our third aim was to extend our findings of increased Bcl6 target miRNA (miR-21) in mouse models of Th2 inflammation to assess its relevance as a diagnostic biomarker for human Th2 diseases like asthma and eosinophilic esophagitis.

Treg cells demonstrate considerable plasticity and remodeling in inflammatory contexts. Since the Bcl6-deficient mice exhibit Th2 inflammatory pathology, the Bcl6-deficient Treg phenotype, function and stability are likely to be modulated by the inflammatory milieu. Hence, the fourth aim focused on characterization of a Bcl6-deficient Foxp3 dual reporter mouse strain that allows

assessment of stability of Bcl6-deficient Treg phenotype. In addition to understanding the extent of remodeling contributed by the Th2 disease, we wanted to assess the effects from loss of Bcl6 specifically in the Treg lineage. Thus the fifth aim encompasses characterization of a Treg-specific conditional mutant mouse strain that could definitively differentiate the intrinsic role of Bcl6 in the Treg lineage and how much that impacts the overall pathology that develops in the Bcl6-deficient mice.

## MATERIALS AND METHODS

### Mice

Bcl6-deficient mice on a mixed C57BL/6-129Sv background have been previously described (Dent *et al.*, 1998b; Dent *et al.*, 1997). Bcl6-deficient mice were used between 5 and 10 weeks of age, and mice used were active and relatively healthy. Foxp3-gfp mice where an IRES element links GFP expression to the Foxp3 transcript were obtained from Jackson labs (strain B6.Cg-*Foxp3tm2Tch/J*; Bar Harbor, ME). Bcl6-deficient mice were mated onto the Foxp3gfp background. Rag1-deficient, IL-10-deficient, Foxp3-gfp-cre (Foxp3<sup>cre</sup>), Foxp3-deficient (scurfy), Rosa26-YFP reporter and C57BL/6 congenic CD45.1<sup>+</sup> (BoyJ) wild-type mice were obtained from Jackson labs. The wild-type BoyJ mice were also bred onto the Foxp3gfp background. Bcl6/Tcra double-deficient mice have been previously described (Toney *et al.*, 2000). Wild-type and Bcl6-deficient Foxp3gfp mice were genotyped by PCR as previously described. Bcl6<sup>fl/fl</sup> mice were generated in the Indiana University School of Medicine (IUSM) Transgenic and Knockout facility. Bcl6<sup>fl/fl</sup> mice were mated to Foxp3<sup>cre</sup> mice to generate Bcl6<sup>fl/fl</sup> Foxp3<sup>cre</sup> mice. Mice were bred under specific pathogen-free conditions at the laboratory animal facility at IUSM and were handled according to protocols approved by the IUSM Animal Care and Use Committee.

## **Human patient samples**

The collection and analysis of control, EoE patient, asthma and Crohn's disease patient samples were approved by the Institutional Review Board of Indiana University and required parental consent for samples from infants. Details on the patient populations have been described previously (Subbarao *et al.*, 2011; Tepper *et al.*, 2008).

## **Murine helper T cell differentiation and Treg cell assays**

Naïve T cells (CD4<sup>+</sup>CD62L<sup>+</sup>) were purified from lymph nodes and spleen using magnetic beads (Miltenyi Biotech). Naïve CD4<sup>+</sup> T cells (1x10<sup>6</sup> cells/ml in DMEM medium supplemented with 10% FCS (Atlanta Biologicals), 2 mM glutamine, 100 units/ml Penicillin-Streptomycin, MEM non-essential amino acids, 25 mM HEPES and 55 µM 2-mercaptoethanol (Gibco) were activated with plate-bound anti-CD3 (5 µg/ml; 145-2C11; BD Biosciences) and anti-CD28 (10 µg/ml; 37.51; BD Biosciences) and polarized under Th0 (with no added cytokines), Th1 (20 ng/ml IL-12 and 10 µg/ml anti-IL-4), Th2 (20 ng/ml IL-4 and 10 µg/ml anti-IFNγ), iTreg (5 ng/ml TGFβ, 10 µg/ml anti-IFNγ and 10 µg/ml anti-IL-4), Th17 (20 ng/ml IL-6, 5 ng/ml TGFβ, 10 µg/ml anti-IFNγ and 10 µg/ml anti-IL-4), Th9 (10 ng/ml IL-4, 2 ng/ml TGFβ, 10 µg/ml anti-IFNγ and 10 µg/ml anti-IL-4), Tfh (20 ng/ml IL-6, 20 ng/ml IL-21, 10 µg/ml anti-IFNγ, 10 µg/ml anti-IL-4 and 10 µg/ml anti-TGFβ) differentiation conditions. After 5 days of culture, cells were re-stimulated with plate-bound anti-CD3 (10 µg/ml) for 12-16 hours prior to harvest. Recombinant mouse IL-4 was purchased from R&D Systems and Abs to CD3 and CD28 from



BD Biosciences. Recombinant human IL-2 was obtained from the Biological Resources Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute-Frederick Cancer Research and Development Center.

For Treg assays, CD4<sup>+</sup>CD25<sup>+</sup> T cells were purified from wild-type and Bcl6-deficient Foxp3gfp mice using magnetic beads, followed by FACS sorting for pure GFP<sup>+</sup> Tregs using FACS Aria cell sorter (Becton Dickinson). The CD4<sup>+</sup>CD25<sup>-</sup> T cell fraction from magnetic bead isolation was used as responder T cells (Tresps). Antigen-presenting cells (APCs) were prepared from the spleens of Tcra-deficient mice. For Treg suppression assays, wild-type or Bcl6-deficient Tresps (50x10<sup>3</sup>/well) were separately co-cultured with wild-type or Bcl6-deficient Tregs at different ratios as indicated along-with Mitomycin-C (20 µg/ml) treated APCs (50x10<sup>3</sup>/well) plus anti-CD3 (2 µg/ml) or 10 µg/ml Ova for 72 hrs. Plates were pulsed with 1 µCi of [<sup>3</sup>H] thymidine at 48 hrs of culture and cell proliferation in triplicate cultures was measured using a scintillation counter.

### **Antibodies and FACS analysis**

Flow cytometry analysis of intracellular transcription factors and cytokines was performed by staining the cells with flouochrome-conjugated anti-Foxp3 (FJK-16a; eBioscience), anti-Helios (22F6; BioLegend), anti-Gata3 (TWAJ; eBioscience) and anti-IL-4 (BD Biosciences) using the mouse Regulatory T cell staining kit (eBioscience). Cells were first stained with antibodies for the desired cell surface markers – CD4 (RM4-5; BD Biosciences) and CD25 (PC61.5; eBioscience), followed by permeabilization with Fixation/Permeabilization buffer

and intracellular staining in Permeabilization buffer. For the bone marrow chimera experiments, cells were stained for Treg markers (CD4, CD25) and CD45.1 (A20; BD Biosciences). For discrimination of GFP versus YFP signals in the BRFC mice, 510-nm/ 20-nm filters (505LP dichroic mirror, GFP) and 525-nm/50-nm filters (535LP dichroic mirror, YFP) were used (Zhou *et al.*, 2009c). For analysis of Tfh cells, spleen cells were stained for CD4 (RM4-5; BioLegend), CXCR5 (2G8; BD Biosciences), PD-1 (29F.1A12; BioLegend) and ICOS (7E.17G9; eBioscience) expression. GC B cells were stained for B220 (RA-3-6B2; BioLegend), GL-7 (GL7; BD Biosciences) and CD95 (Jo2; BD Biosciences) and plasma cell were stained for CD138 (281-2; BD Biosciences). Flow analysis was performed on a FACSCalibur (4 color) and LSR 561 analyzer (7-8 color) and data analyzed using the FLOWJO software (Tree Star, Inc.).

### **Induction of experimental colitis**

Un-fractionated CD4<sup>+</sup> T cells (Tresps) ( $1 \times 10^6$ ) from Il10-deficient mice isolated using magnetic bead selection were adoptively transferred intra-peritoneally (i.p.) into Rag-deficient mice to induce a strong Th1-mediated colitogenic response in the recipients 4-8 wks post-transfer (Ostanin *et al.*, 2009). Along with the transfer of Il10-deficient Tresps, two cohorts of Rag-deficient recipients were injected i.p. with FACS-sorted CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs ( $250 \times 10^3$ ) from Bcl6-deficient or wild-type Foxp3gfp mice. The recipient mice were monitored for signs of intestinal pathology and weight loss over a period of 4-5 wks, following which

mice were sacrificed to assess severity of colitis using the parameters of percent weight loss, changes in colon length and colon histology scores.

Colon sections were stained with hematoxylin and eosin (H&E) and colitis severity was graded in a blinded fashion on a scale of 0-6 as described: 0 – Normal crypt architecture and occasional cell infiltration, 2 – Irregular crypt architecture and increasing number of cells in lamina propria (LP), 4 – Moderate crypt loss (10-50%) and confluence of cells extending to sub-mucosa, 6 – Severe crypt loss (50-90%) and transmural extension of infiltrate.

### **Induction of experimental allergic airway inflammation**

Both wild-type B6 female mice (recipients) as well as the Bcl6-deficient and wild-type Foxp3gfp mice (Treg donors) were sensitized intra-peritoneally (i.p.) with Ova (Sigma) adsorbed to alum (Sigma) at a dose of 20 µg Ova/2 mg alum on days 0 and 7 of the protocol (Chang *et al.*, 2010). On day 14, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs were FACS-sorted from Ova-sensitized Bcl6-deficient and wild-type Foxp3gfp mice and then injected i.p. (350 x 10<sup>3</sup> cells/mouse) into the sensitized wild-type B6 female recipients. After 3 hrs following immunization, recipient mice were then challenged intranasally with Ova for 5 consecutive days (100 µg/ day). Mice were sacrificed by i.p. injection of pentobarbital (5 mg/mouse) 48 hrs after the final intranasal challenge. The trachea was cannulated and lungs were lavaged three times with 1 ml PBS to collect the bronchoalveolar lavage (BAL). Cells recovered in BAL fluid and the lung mediastinal lymph nodes (MLNs) were counted with a hemocytometer. Eosinophils, neutrophils, T cells, B cells and

mononuclear cells in the BAL fluid were distinguished by cell size and by expression of CD3, B220, CCR3, CD11c and major histocompatibility complex class II, analyzed by flow cytometry as described (Chang *et al.*, 2010; van Rijt *et al.*, 2004). For quantitative PCR analysis, lung tissues were homogenized in a tissue lyser (Qiagen) and RNA isolated with an RNeasy kit (Qiagen) was used for synthesis of cDNA for subsequent analysis. Paraffin-embedded sections were stained with H & E for evaluation of the infiltration of inflammatory cells by light microscopy.

Airway hyper-reactivity to methacholine challenge was determined 24 hrs after the final intranasal challenge. Noninvasive unrestrained whole-body plethysmography (Buxco Research Systems) was used to record airway responsiveness with the dimensionless parameter 'enhanced pause' for estimation of total pulmonary resistance, an indicator of broncho-constriction. Mice were placed in whole-body plethysmographs and baseline measurements were recorded. Saline was administered by nebulization for 2 min, followed by increasing doses of methacholine, and the enhanced-pause parameter was recorded over 5 min.

### **Generation of bone marrow chimeras**

Donor wild-type BoyJ (CD45.1<sup>+</sup>) and Bcl6-deficient (CD45.1<sup>-</sup>) mice on Foxp3gfp background and Foxp3-deficient mice were euthanized with CO<sub>2</sub> asphyxiation and cervical dislocation, and femurs and tibias were removed aseptically. Bone marrow (BM) was flushed with DMEM complete media. Recipient Rag-deficient

mice were sub-lethally irradiated (350 Gy) 16-24 hrs prior to re-constitution. The recipients were then reconstituted with wild-type and Foxp3<sup>KO</sup> BM (WT:Foxp3<sup>KO</sup> chimeras), Bcl6-deficient and Foxp3<sup>KO</sup> BM (KO:Foxp3<sup>KO</sup> chimeras) or wild-type and Bcl6-deficient Foxp3gfp BM (WT:KO chimeras) ( $10 \times 10^6$ ) by intravenous injections (i.v.). Flow analysis was performed to assess satisfactory reconstitution of the lymphocyte and myeloid compartments after 4-5 months. Mice were immunized with OVA/Alum i.p. two weeks prior to FACS sorting of the wild-type (CD45.1<sup>+</sup>) and Bcl6-deficient (CD45.1<sup>-</sup>) CD25<sup>+</sup>Foxp3<sup>+</sup> (Tregs) and CD25<sup>-</sup>Foxp3<sup>-</sup> (Tconv) cells for quantitative PCR analysis.

#### **Affymetrix Microarrays and qRT-PCR**

Total RNA was extracted from FACS-sorted CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs from Bcl6-deficient and wild-type Foxp3gfp mice following 16 hr activation *in vitro* with plate-bound anti-CD3 (5 µg/ml) and anti-CD28 (10 µg/ml) using the RNeasy Mini kit, according to the manufacturer's protocol (Qiagen). The microarray studies were carried out using the facilities of the Center for Medical Genomics at Indiana University School of Medicine which process the samples employing the protocols recommended by Affymetrix in their GeneChip® Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Biotinylated cRNA was hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 arrays. Data analysis was performed using the MeV software.

To validate the expression array data, qPCR was performed on independently prepared Tregs from Bcl6-deficient and wild-type Foxp3gfp mice. PCR primer sequences are listed in Table 1.

### **Gene expression analysis**

Total cellular RNA was prepared using the Trizol method (Life Technologies) and cDNA prepared with the Transcriptor First Strand cDNA synthesis kit (Roche). Quantitative PCR reactions were run by assaying each sample in triplicates using the Fast Start Universal SYBR Green Mix (Roche Applied Science) with a Stratagene Mx3000P Real-Time QPCR machine. Samples with limiting RNA were assessed for gene expression using Taqman assays (ABI). Levels of mRNA expression were normalized to *β-tubulin* mRNA levels, and differences between samples analyzed using the  $\Delta\Delta CT$  method. PCR primer sequences and Taqman assay IDs are listed in Table 1.

<b>Genes</b>	<b>Taqman assay IDs</b>
<b><i>Tubb5</i></b>	Mm00495804_m1
<b><i>Spry1</i></b>	Mm00839969_g1
<b><i>Pten</i></b>	Mm00477208_m1
<b><i>Pdcd4</i></b>	Mm01266062_m1
<b><i>Btg2</i></b>	Mm00476162_m1
<b><i>Il12a</i></b>	Mm00434169_m1
<b>miR-21</b>	000397
<b>miR-22</b>	000398
<b>miR-146b</b>	002453
<b>miR-16</b>	002489
<b>U6</b>	001973
<b>sno202</b>	001232
<b>sno234</b>	001234

<b>Genes</b>	<b>SYBR Green PCR primer sequences</b>
<b><i>β-tub</i></b>	F: 5'-CTGGGAGGTGATAAGCGATGA-3' R: 5'-CGCTGTCACCGTGGTAGGT-3'
<b><i>Foxp3</i></b>	F: 5'-CCCTGCCCTTGACCTCAA-3' R: 5'-GCCTCAGTCTCATGGTTTTGG-3'
<b><i>Il4</i></b>	F: 5'-ACAGGAGAAGGGACGCCAT-3' R: 5'-GAAGCCCTACAGACGAGCTCA-3'
<b><i>Il5</i></b>	F: 5'-GCAGCTGGATTTTGGAAA-3' R: 5'-GTGGCTGGCTCTCATTACACA-3'
<b><i>Il13</i></b>	F: 5'-CGCAAGGCCCCCACTAC-3' R: 5'-AAAGTGGGCTACTTCGATT-3'
<b><i>Il10</i></b>	F: 5'-GGTTGCCAAGCCTTATCGGA-3' R: 5'-ACCTGCTCCACTGCCTTGCT-3'
<b><i>Tgfβ1</i></b>	F: 5'-GCAGTGGCTGAACCAAGGA-3' R: 5'-AGCAGTGAGCGCTGAATCG-3'
<b><i>Gzmb</i></b>	F: 5'-CCCAGGCGCAATGTCAAT-3' R: 5'-CCCCAACCAGCCACATAGC-3'
<b><i>Ccr8</i></b>	F: 5'-TGTGGCTGGCTGCTGTCA-3' R: 5'-TCAGAGGCCACTTGGTAAAAAAC-3'
<b><i>Tnfa</i></b>	F: 5'-CACAAGATGCTGGGACAGTGA-3' R: 5'-TCCTTGATGGTGGTGCATGA-3'
<b><i>Gata3</i></b>	F: 5'-GAAGGCATCCAGACCCGAAAC-3' R: 5'-ACCCATGGCGGTGACCATGC-3'
<b><i>Ifny</i></b>	F: 5'-TCAAGTGGCATAGATGTGGAAGAA-3' R: 5'- TGGCTCTGCAGGATTTTCATG-3'
<b><i>Smad7</i></b>	F: 5' - CAGCACTGCCAAGCATGGT-3' R: 5'- ACCGAAACGCTGATCCAAAG-3'
<b><i>Bcl6</i></b>	F: 5' - GAAGACCCACACTCGAATTCAT-3' R: 5'- CCCCACAGGTTTCACATTTGT-3'

**Table 1: Taqman probe IDs and SYBR Green Primer sequences used for quantitative real time-PCR.**



## **Retroviral transductions**

Naïve T cells ( $CD4^+CD62L^+$ ) prepared from wild-type C57BL/6 or Stat6-deficient mice using magnetic beads were activated *in vitro* with plate-bound anti-CD3 (5  $\mu$ g/ml) and anti-CD28 (10  $\mu$ g/ml) for 24 hrs. Cells were then transduced by spin infection with bicistronic retroviral vector (RV) supernatants encoding Bcl6 and H2K<sup>k</sup> or Gata3 and hCD4 (Chang *et al.*, 2005; Kusam *et al.*, 2003). Naïve T cells activated similarly under Th0 and Th2 differentiation conditions were infected with retroviral supernatant encoding miR-21 and H2K<sup>k</sup>. Wild-type Treg cells were similarly transduced with retroviral supernatant encoding miR-21 and H2K<sup>k</sup>. Second or third day following transduction, cells infected with H2K<sup>k</sup> RVs were stained with biotin-anti-H2K<sup>k</sup> and streptavidin-APC, while those infected with hCD4 RVs were stained for anti-hCD4-PE and then FACS-sorted based on APC or PE expression, respectively. The sorted RV<sup>+</sup> T cells were re-stimulated *in vitro* with anti-CD3 and anti-CD28 for 4-6 hrs for gene expression analysis or overnight for cytokine measurements.

## **Reporter assays**

Jurkat T cells ( $10 \times 10^6$  cells/250ul) were electroporated as described (Vasanwala *et al.*, 2002) in serum-free RPMI 1640 medium with an IL-5 promoter-driven luciferase reporter vector or Foxp3 promoter (10  $\mu$ g) along with expression constructs (10  $\mu$ g) for CXN, CXN-Gata3, CXN-Bcl6 or CXN-Gata3 plus CXN-Bcl6 at the concentrations listed. Similarly, wild-type Foxp3 promoter-driven luciferase reporter vector and a mutated version (with the two GATA sites deleted) was

tested in Jurkat T cells with titrations of CXN-Gata3 (0-10  $\mu$ g). After electroporation, cells were re-suspended in RPMI media supplemented with 10% FCS and rested overnight. For experiments with co-transfection of full-length, SB1 and SB2 miR21 promoter constructs (1  $\mu$ g) and expression constructs for CXN or CXN-Bcl6 (1  $\mu$ g),  $1 \times 10^6$  cells Jurkat T cells were transiently transfected with X-tremeGENE HP DNA transfection reagent (Roche), according to the manufacturer's protocol. Luciferase measurements were performed 24 hrs after transfection following 6 hr activation of cells with PMA (10 ng/ml) and Ionomycin (0.3  $\mu$ M) using Luciferase Assay System (Promega).

#### **MicroRNA profiling and qRT-PCR assessment of microRNAs**

RNA was extracted from FACS-sorted CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs from wild-type and Bcl6-deficient Foxp3gfp mice following 16 hr activation *in vitro* with anti-CD3 (5  $\mu$ g/ml) and anti-CD28 (10  $\mu$ g/ml) using MiRNeasy Mini kit, according to manufacturer's protocol (Qiagen). The quality of the total RNA was verified by an Agilent 2100 Bioanalyzer profile. Total RNA (140 ng) was tagged with fluorescent label. The samples were hybridized to the miRCURY<sup>™</sup> LNA array version 11.0 (Exiqon, Denmark), which contains capture probes targeting all miRNAs for human, mouse or rat registered in the miRBASE version 13.0 at the Sanger Institute.

Validation of the differentially expressed microRNAs from the microarray dataset, assessment of microRNA expression profile of the total lungs and esophageal biopsies as well as from mouse and human serum was performed

using TaqMan microRNA assays (Applied Biosystems), according to the manufacturer's protocol. Normalization was performed using sno202, sno234 and U6 as controls, with U6 as the sole control for samples with limiting RNA. Quantitative real-time PCR was performed by assaying each sample in triplicates, including no-template controls, on a Stratagene Mx3000P real-time PCR system. Relative expression was calculated using the delta-delta CT (ddCT) method, as previously described.

### **Cloning of mmu-microRNA-21 and miR-21 reporter vectors**

The miR-21 gene representing the primary transcript (~300 bp) was PCR amplified from mouse genomic DNA and cloned into retroviral vector co-expressing H2K<sup>k</sup> via *EcoRI* (restriction enzyme). The microRNA is expressed as a partial-primary microRNA transcript, transcribed from the retroviral LTR.

F (miR-21): 5'-AATT-GAATTC-GGTACC-TTGGCATTAAAGCCCCAGCAAACC-3'

R (miR-21): 5'-AATT-GAATTC-TCCAAGTCTCACAAGACATAAGGACC-3'

Full-length mouse miR-21 promoter region was PCR amplified from mouse genomic DNA and was inserted using *Mlu-I* and *Xho-I* restriction enzyme sites into the pGL3-basic vector (Promega). SB1 and SB2 mutations in the miR-21 promoter region were created by insertion of restriction enzyme sites in the respective STAT/Bcl6 binding site by amplifying with mutated primer sequences, followed by insertion into the pGL3-basic vector. Primer sequences listed in table 2. All constructs were sequenced prior to their application to confirm construct integrity.

MIR21 PROMOTER CONSTRUCTS	PRIMER SEQUENCES
<b>Full-length promoter</b>	Full-length Forward: 5'-AAAAAA ACGCGT GGT TTT GTG GCA AGG TCT CCG TGT-3' Full-length Reverse: 5'-AAAAAA CTCGAG TGT CAG GAG GGC GAG GCT AGT GCA-3'
<b>SB1-mutated promoter</b>	SB-mut1 Forward: 5'-TTAA ACCAG TTCTT <b>GAATT</b> CACTA GTGGT GATAA ATGTG GGACT <b>TCTCA</b> <b>GAAGT</b> CATTG ATTTT-3' SB-mut1 Reverse: 5'-AAAAT GAATG <b>ACTTC</b> <b>TGAGA</b> AGTCC CACAT TTATC ACCAC TAGTG <b>AATTC</b> <b>AAGAA</b> CTGGT TTAA-3'
<b>SB2-mutated promoter</b>	SB-mut2 Forward: 5'-TTAA ACCAG TTCTT <b>ACAGG</b> <b>AACTA</b> GTGGT GATAA ATGTG GGACT <b>TAGGC</b> <b>CTAGT</b> CATTG ATTTT-3' SB-mut2 Reverse: 5'-AAAAT GAATG <b>ACTAG</b> <b>GCCTA</b> AGTCC CACAT TTATC ACCAC TAGTT <b>CCTGT</b> <b>AAGAA</b> CTGGT TTAA-3'

**Table 2: Primer sequences for miR-21 promoter reporter constructs**

### **Overexpression and inhibition of miR-21**

Naïve T cells activated under Th0 conditions (with nothing added) were treated with 1  $\mu$ M control (scrambled oligo), miR-21 mimic (double stranded RNA oligo) and antagomiR (single stranded DNA oligo) (Exiqon). The oligos were cholesterol linked that enabled efficient delivery into cells, without the need of any transfection protocol. Gene expression was assessed 12-16 hrs following treatment. For assessment of cytokine productions, cells were treated with the oligos over a 5-day period, following which the cultures were re-stimulated with anti-CD3 (10  $\mu$ g/ml) overnight to obtain cell-free supernatants for ELISA.

### **In situ hybridization**

In situ hybridizations were performed in 8  $\mu$ M cryosections from lungs of mice in the airway inflammation experiment using the miRCURY LNA microRNA ISH Optimization kit 2 (miR-21), according to the manufacturer's protocol (Exiqon). In brief, the FFPE slides were rinsed and digested with Proteinase K for 12 mins at 37°C. After protease digestion, the digoxin-labeled LNA-scrambled control probe and LNA miR-21 antisense probe (Exiqon) were hybridized to the slides at 52°C for 6 hrs. Following post-hybridization washes with SSC buffer at 47°C, 100  $\mu$ l of rabbit anti-digoxin (Sigma-Aldrich) Ab, diluted 1/2000 was applied to the slides for 1 hr at room temperature. The slides were rinsed and then incubated with 100  $\mu$ l anti-rabbit alkaline phosphatase and TNBS substrate for 2 hr at 30°C. Slides were counterstained with Nuclear Fast Red (Polyscientific), coverslipped, and mounted for viewing.

### **Assessment of myocarditis**

Heart sections from  $Bcl6^{neofl/neofl}Foxp3^{cre}$  and  $Bcl6^{neofl/neofl}$  mice were stained with hematoxylin and eosin (H&E) and severity of myocarditis was graded in a blinded fashion on a scale of 0-4 as described: 0 – No cell infiltration, 1 – Sparse cell infiltration, 2 – Small foci of cell infiltration, 3 – Large foci of cell infiltration and moderate loss (10-50%) of architecture, 4 – Massive cellular infiltration and severe loss (50-90%) of myocardial architecture.

### **Cytokine secretion analysis**

Cytokines were measured from 24 hr activated cell-free supernatants by ELISA. IL-4, IL-5, IFN $\gamma$  and IL-17 ELISA reagents were purchased from BD Biosciences; IL-13 reagents purchased from R&D systems. To test for cytokine secretion, 100  $\mu$ l/well of 1:500 dilution of the capture antibody in Coating Buffer was used to coat a 96 well immunosorbent plate (Thermo Scientific). Plates were sealed and incubated overnight at 4 $^{\circ}$  C and then washed three times next day in ELISA wash buffer (PBS with 0.05% Tween-20). Plates were next blocked with 200  $\mu$ l/well Assay Diluent (PBS with 10% FBS) for minimum 2 hours at room temperature. Cytokine standards, sample supernatants and controls diluted in Assay Diluent (100  $\mu$ l/well) were added and incubated for 2 hours at room temperature or overnight at 4 $^{\circ}$  C. Plates were washed five times in ELISA wash buffer and 100  $\mu$ l/well of Working Detector (1:500 dilution of Detection antibody and 1:250 dilution of Avidin-HRP reagent) was incubated for 1 hour at room temperature. Plates were washed seven times in ELISA wash buffer and 100  $\mu$ l/well of

Substrate Solution (Tetramethybenzidine, TMB and Hydrogen Peroxide) was incubated for 5-30 mins at room temperature in dark and cytokine levels were determined following addition of 50  $\mu$ l/well of Stop Solution (2N  $\text{H}_2\text{SO}_4$ ) by measuring the absorbance at 415 nm (BIO-RAD iMark Microplate Reader).

The concentration of IL-4, IL-5, IL-13, IFN $\gamma$  and IL-17A in samples with limiting cytokine levels was measured by multiplex analysis with the Milliplex MAP Kit according to the manufacturer's protocol (Millipore) (Chang *et al.*, 2010). Cytokine concentrations were calculated with Bio-Plex Manager 2.3 software with a five-parameter curve-fitting algorithm applied for standard-curve calculations.

### **Statistical analysis**

*p* values were calculated using Students' T-test or One-way analysis of variance with Tukey's post test (for multiple sample comparisons). All calculations were done using GraphPad Prism software. A *p* value < 0.05 was considered to show a significant difference.

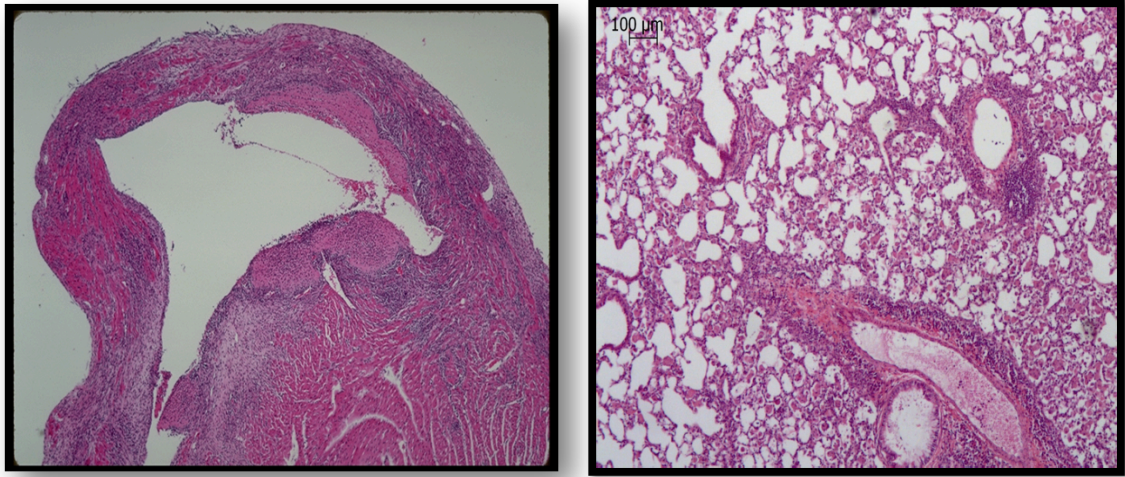
## RESULTS

### **Part I: Bcl6 limits the Th2 inflammatory activity of Treg cells by the control of Gata3 function**

#### **T cell-dependent spontaneous autoimmune disease in Bcl6-deficient mice**

Approximately 80% of the Bcl6-deficient mice develop spontaneous Th2-type inflammatory disease, commonly manifested as severe myocarditis and frequently accompanied by pulmonary vasculitis (Figure 4) and most of these mice die by 6-12 weeks of age (Dent *et al.*, 1998a; Dent *et al.*, 1997; Ye *et al.*, 1997). Although the disease severity correlates well with the observed growth retardation (nearly one-third or half the body weight of wild-type littermates) and the apparent moribund phenotype of the Bcl6-deficient mice, a small percentage (~10-20%) of these mice appear grossly normal and display relatively mild inflammation in heart and/or lungs when assessed by histology. The inflammation is complex in nature, with infiltration of cells from both the lymphoid and myeloid compartments - T cells, macrophages and granulocytes, mainly eosinophils (Toney *et al.*, 2000; Yu *et al.*, 2005). In addition to the eosinophilic infiltration, the infiltrating T cells in the lesions predominantly produce the hallmark Th2 cytokines (IL-4, IL-5 and IL-13), demonstrating the Th2-bias of the inflammation (Dent *et al.*, 1997). To assess the role of T cells in the inflammatory disease, Bcl6/Rag double-deficient and Bcl6/Tcr $\alpha$  double-deficient mice were bred and examined for signs of the hyper-Th2 inflammatory disease (Table 3). Absence of inflammation in Bcl6/Tcr $\alpha$  double-deficient mice highlights a key role for  $\alpha\beta$ -T cells in mediating this spontaneous inflammation.





**Figure 4: Spontaneous myocarditis and pulmonary vasculitis that develops in Bcl6-deficient mice.**  
Heart (Left) and lung (Right) histology as assessed by hematoxylin & eosin (H& E) staining of sections.

Mouse Strain	Rate of Myocarditis	Rate of Pulmonary Vasculitis
Bcl6-deficient	7/8	5/5
Bcl6/Rag double-deficient	0/4	0/4
Bcl6/Tcra double-deficient	0/8	0/8

**Table 3: Histological assessment of frequency of myocarditis and pulmonary vasculitis in Bcl6-deficient mice on different genetic backgrounds.**

### **Inflammatory disease in Bcl6-deficient mice is reminiscent of pathology in mice with defects in Treg cell lineage**

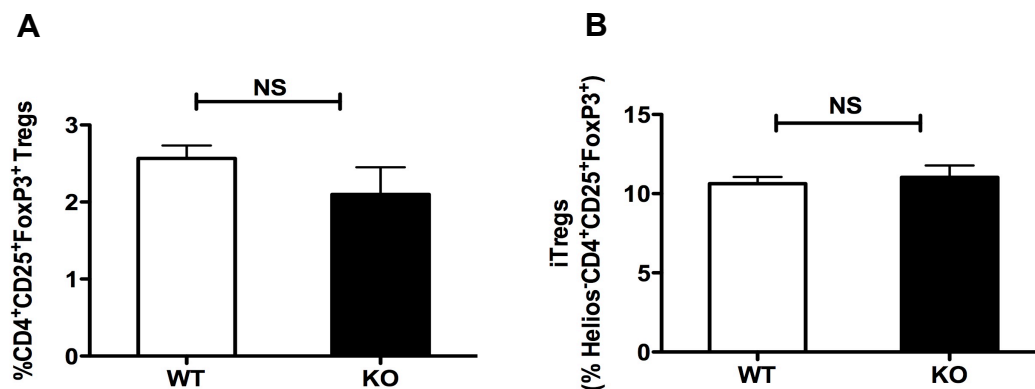
The disease in Bcl6-deficient mice is spontaneous in nature, T cell-dependent and resembles the pathology in mice with Treg defects. Mice lacking genes critical for Treg phenotype and suppressor function (Foxp3, Ctla-4, TGF $\beta$ 1) develop spontaneous autoimmune diseases owing to reduced Treg numbers or altered function. Foxp3-deficient or scurfy mice succumb to a spontaneous T cell-dependent multi-organ autoimmune pathology manifested as diabetes, thyroiditis, hemolytic anemia, hyper-IgE syndrome, dermatitis, splenomegaly, lymphadenopathy and cytokine storm (Brunkow *et al.*, 2001; Godfrey *et al.*, 1994; Wildin *et al.*, 2001). Ctla-4-deficient mice develop an aggressive lympho-proliferative disease manifested as splenomegaly, lymphadenopathy, pancreatitis and myocarditis (Tivol *et al.*, 1995). Similarly, TGF $\beta$ 1-deficient mice develop wasting disease by 1-2 weeks of age and inflammatory lesions in many organs, primarily the heart and lungs (Kulkarni and Karlsson, 1993). Thus, reminiscent of the pathologies noted above, the inflammatory disease in Bcl6-deficient mice may have an autoimmune component and could be ascribed to defective Treg cell numbers or activity. The only difference is that unlike the systemic and multi-organ nature of the pathology noted in Foxp3-deficient, CTLA-4-deficient and TGF $\beta$ 1-deficient mice, the disease in Bcl6-deficient mice is restricted to the heart and the lungs. More recent studies demonstrate that Tregs deficient in key effector transcription factors like T-bet, IRF4 and STAT3 exhibit selective defects in control of Th1,

Th2 and Th17-type inflammatory conditions. T-bet-deficient Tregs fail to control Th1 responses during Mycobacterial infection (Koch *et al.*, 2009), mice with IRF4-deficient Tregs develop Th2-driven inflammation in pancreas, stomach and lungs (Zheng *et al.*, 2009) while mice with Stat3 deletion in Tregs succumb to a Th17-mediated intestinal pathology (Chaudhry *et al.*, 2009). Considering that the pathology in the absence of Bcl6 is mainly Th2-biased (Dent *et al.*, 1997), this may indicate that the deletion of Bcl6 results in a partial defect in Treg function, that manifests as a restricted heart and lung-specific pathology. Thus, Bcl6 may be important to regulate a sub-function of Tregs and their ability to control the Th2-arm of the immune response.

### **Bcl6 deficiency does not alter the Treg cell compartment**

We initially analyzed the composition of the Treg compartment in the Bcl6-deficient mice. CD4<sup>+</sup>CD25<sup>+</sup> T cells from the spleen and lymph nodes of wild-type and healthy Bcl6-deficient mice were stained for Foxp3 expression, and assessed by flow cytometry. As shown in Figure 5A, we did not observe any obvious difference in the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in both types of mice. This is consistent with other reports showing that Bcl6 is dispensable for Treg cell development (Yu *et al.*, 2009). Additionally, we tested if induced Treg (iTreg) development was intact in the absence of Bcl6, by staining CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells for Helios expression. Helios, an Ikaros family transcription factor is preferentially expressed by thymically-derived nTregs, thereby differentiating iTregs as Helios<sup>-</sup>CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells (Getnet *et al.*,

2010; Thornton *et al.*, 2010), although some recent reports have raised concerns about its validity as an nTreg marker (Akimova *et al.*, 2011; Getnet *et al.*, 2010; Gottschalk *et al.*, 2012; Verhagen and Wraith, 2010; Zabransky *et al.*, 2012). Nevertheless, we noted normal percentages of Helios<sup>-</sup>CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells in Bcl6-deficient mice as their wild-type counterparts (Figure 5B). *In vitro* differentiation of naïve T cells to iTregs following treatment with TGFβ is reported to be defective with Bcl6-deficient T cells (Nurieva *et al.*, 2009). This may be attributed to the intrinsic Th2 bias of Bcl6-deficient T cells that antagonistically inhibits iTreg differentiation *in vitro*. The induced Treg subset is essential for control of Th2 inflammatory responses at mucosal sites in mice as they age (Josefowicz *et al.*, 2012b). However, the pathology noted in Bcl6-deficient mice develops at an early age and is specific to the heart and lungs and not observed at mucosal environments. Thus, the pattern of pathology and the data with Helios as an nTreg marker can rule out potential defects in iTregs contributing to the disease in Bcl6-deficient mice.



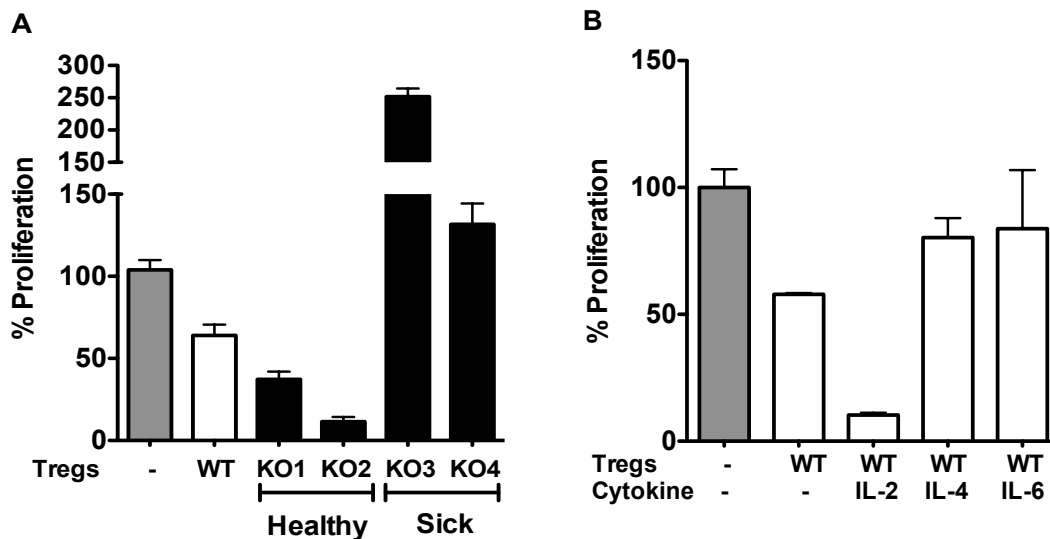
**Figure 5: Treg numbers remain intact in the absence of Bcl6.**

Analysis of Treg cell compartment in the peripheral lymphoid organs of Bcl6-deficient (KO) mice (black bars) and their wild-type (WT) littermates (white bars). The frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs **(A)** and Helios<sup>-</sup>CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> (iTregs) **(B)** was quantified by flow analysis. N = 3 per group (p=0.30 (NS) for CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells and p=0.67 (NS) for iTregs. NS = non-significant (p > 0.05, Student's t-test).

## **Bcl6-deficient Tregs lack anergy, but are competent at suppressing T cell proliferative responses *in vitro***

Since there was no apparent defect in Treg numbers in the absence of Bcl6, our next step was to perform a detailed characterization of the Bcl6-deficient Treg phenotype and function. Treg function is classically assessed *in vitro* using a standard Treg suppression assay, wherein Tregs and responder T cells (Tresps) are cultured at varying ratios, in the presence of soluble anti-CD3 and mitomycin-treated APCs. Our initial attempts to isolate Tregs for these assays involved using magnetic bead-based separation kits to isolate CD4<sup>+</sup>CD25<sup>+</sup> Tregs from the spleen and lymph node preparations of wild-type and Bcl6-deficient mice. Bcl6-deficient Tregs derived from such preparations showed a striking segregation in suppressor function, based on the disease severity. Thus, Tregs isolated from the healthy Bcl6-deficient mice retained normal suppressor function, while Tregs derived from sick Bcl6-deficient mice (assessed by growth retardation and organ histology) were severely defective in controlling T cell proliferative responses *in vitro* (Figure 6A). This suggested that the magnetic bead-based isolation strategy allows purification of Tregs based on CD25 expression and does not control for the percentage of Foxp3<sup>+</sup> cells within the CD25<sup>+</sup> fraction. Any contaminating CD25<sup>+</sup>Foxp3<sup>-</sup> activated effector T cells in the preparations can interfere with the suppression activity of Tregs. Moreover, Bcl6-deficient mice are already well-characterized to have an abundance of spontaneously activated Th2 cells due to the ongoing Th2 inflammatory disease (Dent *et al.*, 1997). Thus, the higher proportion of contaminating effectors in the

Treg preparations from the sick Bcl6-deficient mice can compromise their activity. This impairment in their function was potentially due to the inflammatory cytokines secreted by the contaminating effector Th2 cells (Goodman *et al.*, 2011; Pace *et al.*, 2005; Pasare and Medzhitov, 2003). Indeed, consistent with reports in the literature wherein pro-inflammatory T cell effector cytokines (IL-4 and IL-6) inhibit Treg suppressor function in various disease settings, we noted reduced suppressor function of wild-type Tregs when treated with these inflammatory cytokines (Figure 6B). On the other hand, IL-2 augmented Treg function (Barron *et al.*, 2010), which fits with the reported role for this cytokine in maintaining Treg homeostasis and functional fitness (Figure 6B).



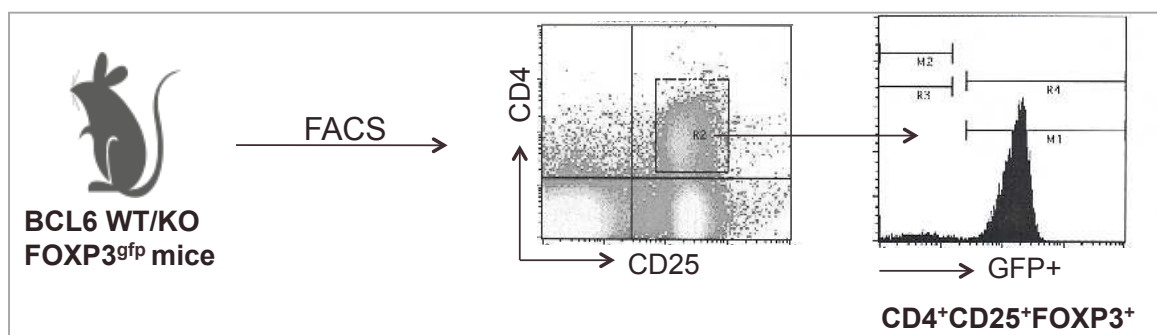
**Figure 6: Treg function modulated by inflammatory cytokines in Bcl6-deficient mice.**

**(A)** CD4<sup>+</sup>CD25<sup>+</sup> Tregs were isolated from wild-type (WT) and 2 healthy (KO1 & KO2) plus 2 sick (KO3 & KO4) Bcl6-deficient mice using MACS beads. Tregs were co-cultured with WT Tresp (CD4<sup>+</sup>CD25<sup>-</sup>) T cells induced by stimulation with mitomycin C-treated spleen cells plus 2 µg/ml α-CD3.

**(B)** WT CD4<sup>+</sup>CD25<sup>+</sup> Tregs were co-cultured with WT Tresp (CD4<sup>+</sup>CD25<sup>-</sup>) T cells in the presence of IL-2 (100 units/ml), IL-4 (50 ng/ml) and IL-6 (20 ng/ml) along-with mitomycin-C treated APCs plus 2 µg/ml α-CD3.

For both **(A)** and **(B)**, wells were pulsed on day 2 of the assay with tritiated thymidine and harvested on day 3. Results shown are normalized proliferation. Results representative of 2 independent experiments.

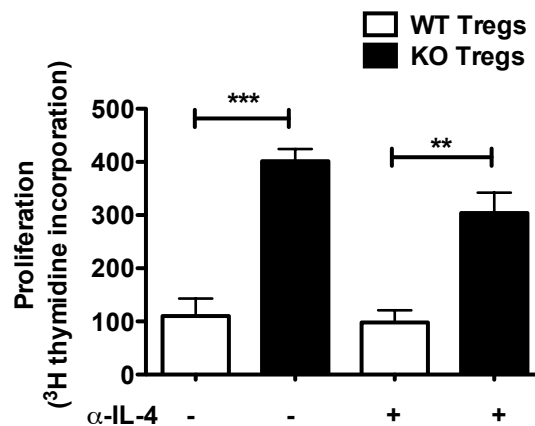
Therefore, in order to obtain pure CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs for functional analysis, we bred the Bcl6-deficient mice onto the Foxp3-gfp knock-in background. Foxp3-gfp mice have an IRES-linked green fluorescent protein (GFP) tagged in the endogenous Foxp3 locus, such that transcription of Foxp3 coincides with that of GFP. Bcl6-deficient mice bred on this background develop the characteristic heart and lung-specific Th2 inflammation as the germ-line Bcl6-deficient mice and thus these mice have been used to sort pure CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs, based on GFP expression for the subsequent studies (Figure 7). Two recent studies have reported that a previously described Foxp3gfp reporter strain in which the GFP is fused to the N-terminal region of Foxp3 is an hypomorph, leading to the development of spontaneous autoimmune pathologies (Bettini *et al.*, 2012; Darce *et al.*, 2012). However, the Foxp3gfp reporter used in our study is a different strain with a bi-cistronic construct of IRES linking Foxp3 and GFP, thus leaving Foxp3 expression intact. Thus, any potential deviation from the hallmark Treg phenotype and function noted on the Bcl6-deficient background is unlikely to be attributable to the inherent defects in the reporter strain.



**Figure 7: Schematic for isolation of pure CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs using Foxp3-gfp reporter mouse model.**

CD4<sup>+</sup>CD25<sup>+</sup> T cells from the spleen and lymph node preparations of wild-type (WT) and Bcl6-deficient (KO) mice on the Foxp3gfp knock-in background are isolated using magnetic bead isolation. These cells are further sort purified using GFP expression to isolate highly pure CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs (~95-99% pure), for functional analysis.

An important feature of Treg cells is their inability to produce IL-2 and proliferate in response to TCR stimulation; this anergic phenotype is essential for maintaining immune homeostasis (Thornton and Shevach, 1998; Walker, 2004). We analyzed the proliferative potential of the pure FACS sorted CD25<sup>+</sup>Foxp3<sup>+</sup> Bcl6-deficient and wild-type Foxp3-gfp Tregs following stimulation with anti-CD3 and anti-CD28. Bcl6-deficient Tregs display a consistent hyper-proliferative phenotype compared to their wild-type counterparts (Figure 8). Since Bcl6-deficient mice have pronounced Th2-differentiation and high levels of IL-4 that is known to negatively impact Treg phenotype and function, we also examined Treg proliferation using an IL-4 blocking antibody. Although there was a slight reduction in proliferation of both wild-type and Bcl6-deficient Tregs with blocking IL-4 antibody, Bcl6-deficient Tregs still displayed a hyper-proliferative phenotype (Figure 8). This may imply an intrinsic role for Bcl6 in regulating Treg activation following TCR stimulation.

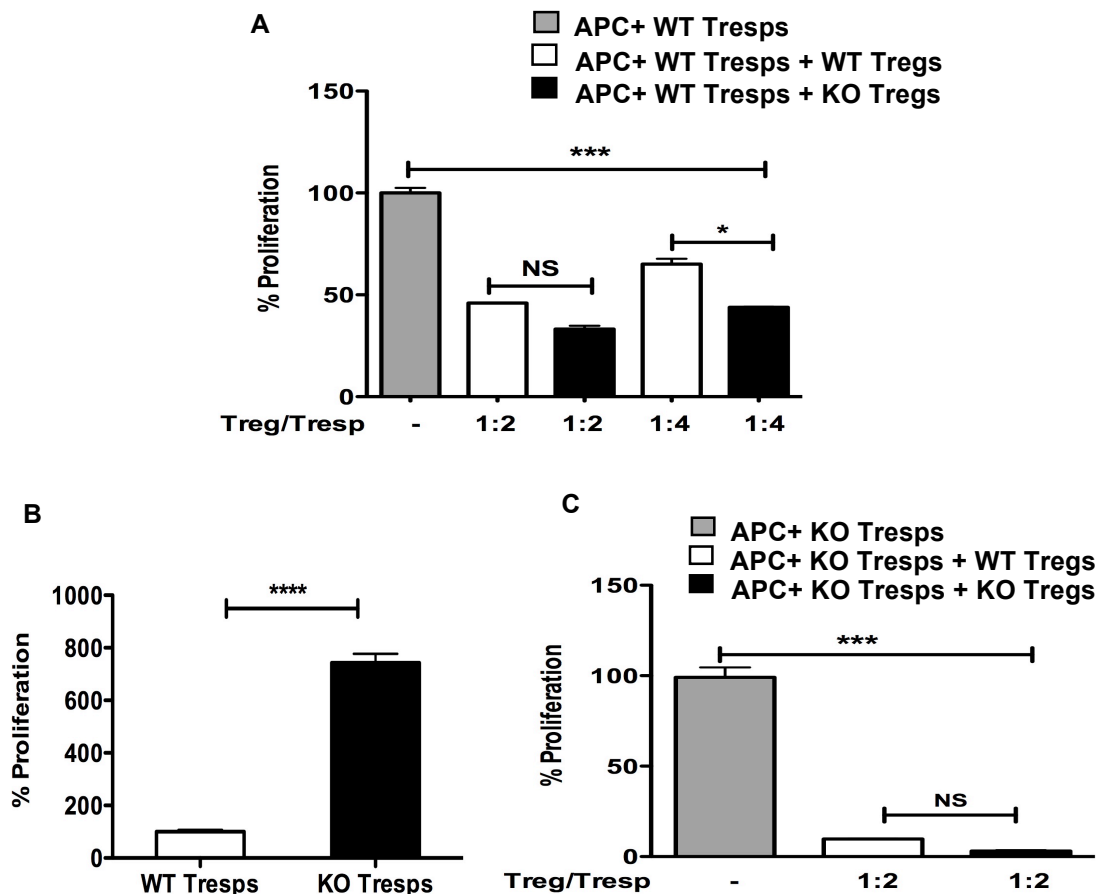


**Figure 8: Bcl6-deficient Tregs lack anergic phenotype *in vitro*.**

Purified CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs were FACS sorted from wild-type (WT) and Bcl6-deficient (KO) Foxp3-gfp mice and activated *in vitro* with 5 α-CD3 and 2 α-CD28 Abs with and without α-IL-4 (10 µg/ml). Wells were pulsed on day 2 of the assay with tritiated thymidine and harvested on day 3. Cell proliferation was determined based on thymidine incorporation. Similar results were observed in 2 independent experiments.



We then tested the functionality of the FACS-sorted CD25<sup>+</sup>Foxp3<sup>+</sup> Bcl6-deficient and wild-type Foxp3-gfp Tregs using the standard *in vitro* Treg suppression assay. We found that Bcl6-deficient Tregs consistently displayed enhanced capacity to suppress wild-type T cell proliferation compared to wild-type Tregs at both the ratios of Tregs/Tresps tested (1:2 and 1:4) (Figure 9A). In certain contexts, Treg cells fail to function due to cytokine-mediated resistance acquired by responder T cells towards Treg suppression (Walker, 2009). In addition, similar to the hyper-proliferative phenotype of Bcl6-deficient Tregs, conventional CD25<sup>-</sup> T cells (Tresps) from Bcl6-deficient mice also exhibit a several fold-increase in proliferative potential than their respective wild-type counterparts (Figure 9B). Interestingly, Bcl6-deficient Tregs were also capable of suppressing the increased proliferation of Bcl6-deficient CD4<sup>+</sup>CD25<sup>-</sup> (Tresps) comparable to wild-type Tregs (Figure 9C), thus demonstrating that Bcl6-deficient Tresps were not resistant to Treg-mediated suppression. Thus, these *in vitro* experiments indicate that despite lacking an anergic phenotype, Bcl6-deficient Foxp3<sup>+</sup> Tregs retain intact suppressor function *in vitro*. Intact *in vitro* suppressor function of Bcl6-deficient Tregs was also recently demonstrated by another group (Chung *et al.*, 2011), which concurs with our results. However, this group used CD4<sup>+</sup>CD25<sup>+</sup> Tregs, which as we have noted above are a mixed population containing activated Foxp3<sup>-</sup> Th2 effectors. Thus, our results using Tregs from Foxp3-gfp mice provide a more refined analysis of the intact functional potency of Bcl6-deficient Tregs *in vitro*.



**Figure 9: Bcl6-deficient Tregs retain intact suppressor function *in vitro*.**

**(A)** Suppression of wild-type T cell proliferative responses by CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs sorted from Bcl6-deficient (KO) (black bars) and wild-type (WT) (white bars) Foxp3-gfp mice following co-culture with wild-type CD25<sup>-</sup>Foxp3<sup>-</sup> (Tresps) cells (grey bar) at different ratios (Treg/Tresp ratios - 1:2 and 1:4).

**(B)** Proliferation of MACS isolated Bcl6-deficient (KO) (black bar) and wild-type (WT) (white bar) CD4<sup>+</sup>CD25<sup>-</sup> T cells (Tresps) following stimulation with anti-CD3 for 72 hrs.

**(C)** Suppression of Bcl6-deficient T cell proliferative responses by CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs sorted from Bcl6-deficient (KO) (black bars) and wild-type (WT) (white bars) Foxp3-gfp mice following co-culture with Bcl6-deficient CD25<sup>-</sup>Foxp3<sup>-</sup> (Tresps) cells (grey bar) (Treg/Tresp ratio-1:2).

Proliferation in the above assays was assessed as [<sup>3</sup>H] thymidine incorporation. Data plotted as percent proliferation is representative of three independent experiments (mean and s.e.m. of triplicate cultures)

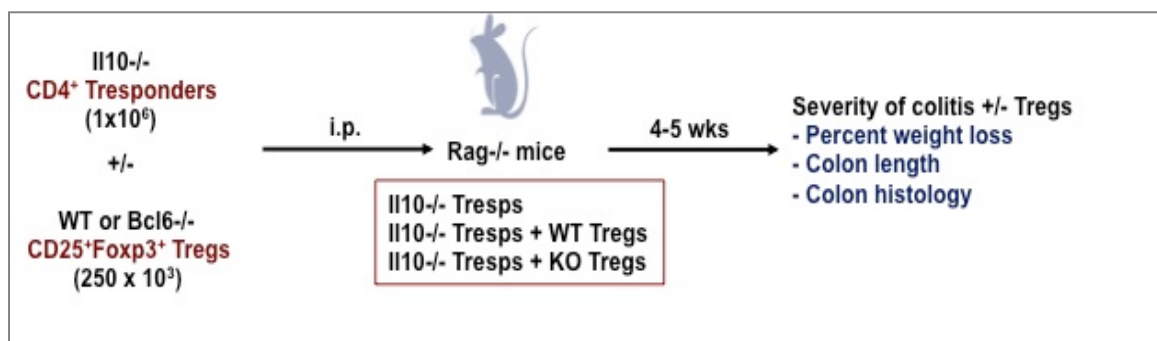
\*p<0.05, \*\*\*p<0.001, \*\*\*\*p<0.0001 (One-way analysis of variance)

### **Treg function *in vitro* does not often correlate with their role *in vivo***

*In vitro* functional assays wherein the Tregs are in close proximity to the APCs and responder T cells may not simulate the setting with pathogenic T cells *in vivo* and thereby have led to discrepancies in interpreting functional potency of Tregs in a variety of disease models. The Treg functional capacity *in vivo* is largely determined by appropriate localization and migratory behavior (Siegmund *et al.*, 2005). Tissue-specific inflammatory diseases in mice lacking specific chemokine receptors (CCR4, CCR7) or adhesion molecules (LFA-1), despite the intact Treg functional competency *in vitro*, highlights the importance of appropriate localization of Tregs in their ability to prevent autoimmunity (Schneider *et al.*, 2007; Wohler *et al.*, 2009; Yuan *et al.*, 2007). In addition to the homing requirements, the *in vivo* inflammatory milieu also impacts the Treg functional competency. The fact that the Treg transcriptional program is not permanently fixed and that Foxp3 gets destabilized under inflammatory settings to allow more regulatory flexibility complicates the situation *in vivo* (Zhou *et al.*, 2009b; Zhou *et al.*, 2009c). Studies *in vitro* assessing the functional efficacy of Bcl6-deficient Tregs have revealed enhanced functional competency compared to their wild-type counterparts, indicative of no intrinsic functional defect. However, the fact that the Bcl6-deficient mice still exhibit pronounced Th2 inflammatory disease of the heart and lungs suggests that a different scenario exists *in vivo*. Therefore, we tested the functional potency of Bcl6-deficient Tregs to control systemic and organ-specific Th1 and Th2 diseases using well-characterized mouse models of inflammation.

## **Bcl6-deficient Tregs are competent at suppressing Th1-mediated colitogenic inflammation *in vivo***

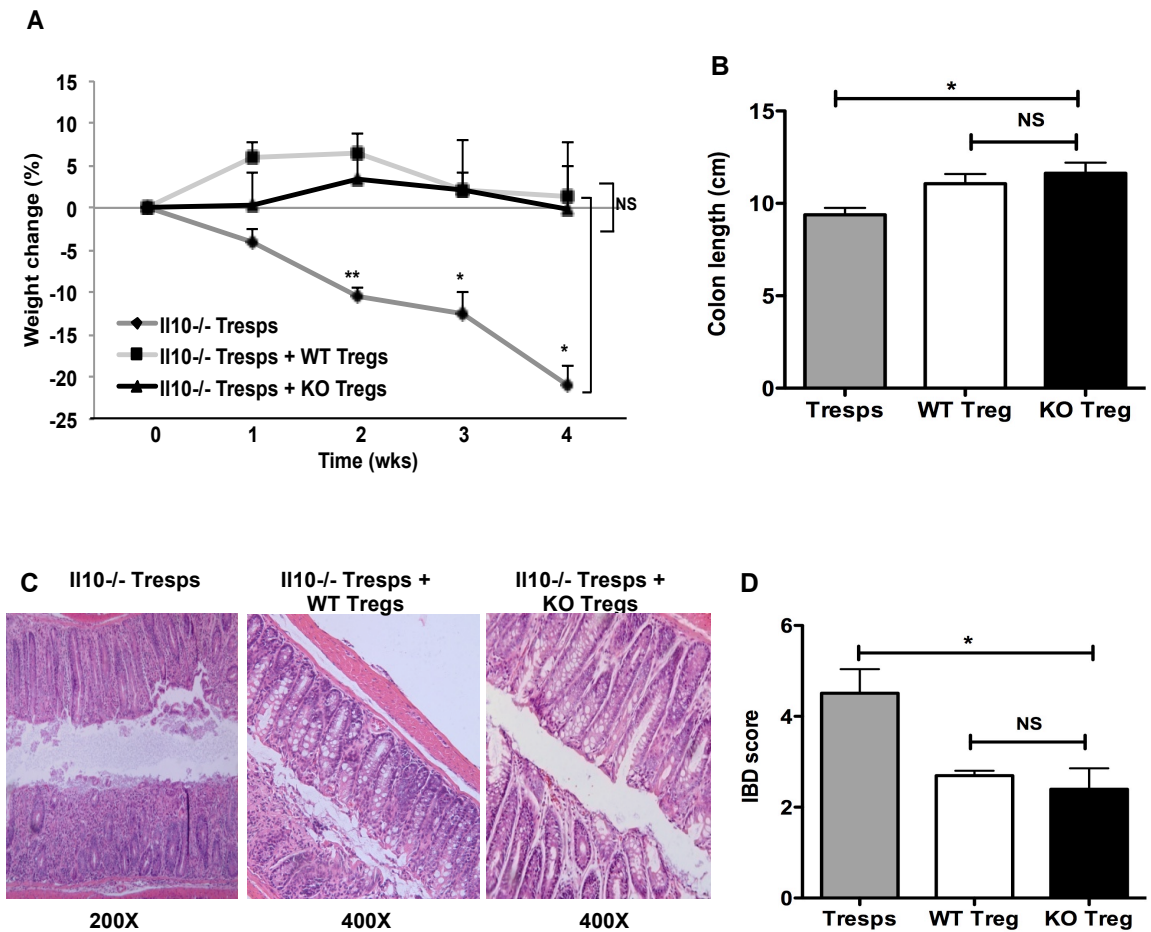
We tested the ability of  $CD4^+CD25^+Foxp3^+$  Bcl6-deficient Tregs to suppress Th1 inflammation *in vivo* in a T cell transfer model of colitis established following adoptive transfer of IL-10-deficient  $CD4^+$  T cells into Rag1-deficient mice (Ostanin *et al.*, 2009) (Figure 10). IL-10 is critical for gut homeostasis and IL-10-deficient mice develop spontaneous enterocolitis due to abnormal immune response to enteric antigens within 2-3 mos. of age (Paul *et al.*, 2012). The disease pathology in this model resembles human inflammatory bowel disease histologically, physiologically and biochemically. Thus, transfer of IL-10-deficient  $CD4^+$  T cells into immune-deficient recipients provokes a strong Th1-mediated colitogenic inflammation 4-8 wks post T cell transfer. This is associated with the characteristic signs of colitis: wasting disease, shortening and thickening of the colon due to the robust inflammation and infiltration of leukocytes, particularly, Th1 cells and their cytokine and chemokine products.



**Figure 10: Schematic for testing Treg suppression of Th1 responses *in vivo* in a T cell transfer model of colitis.**

Colitis was induced following adoptive transfer of IL10-deficient  $CD4^+$  T cells (Trespers) ( $1 \times 10^6$  cells/mouse) i.p. into Rag1-deficient mice. Along-with the Trespers, wild-type or Bcl6-deficient (KO) Tregs ( $250 \times 10^3$  cells/mouse) were injected i.p. into separate cohorts of Rag-deficient mice. Mice were assessed for severity of colitis 4-5 wks later based on percent weight loss, colon length and colon histology.

The experimental protocol involved transfer of IL-10 deficient CD4<sup>+</sup> T cells (Tresps) i.p. into Rag-deficient mice. Along with the Tresps, wild-type and Bcl6-deficient Tregs were injected into separate cohorts of recipients. The recipient mice were then monitored for signs of colitis. We noted that while the control mice that received IL-10-deficient Tresps alone developed weight loss starting from week 1 which progressed over time, the mice receiving either wild-type or Bcl6-deficient Tregs remained healthy and did not exhibit signs of wasting disease (Figure 11A). Also, the colons in the control mice were shorter and thicker, characteristic of severe inflammation, while the colons were relatively normal in mice receiving wild-type and Bcl6-deficient Tregs (Figure 11B). This protection conferred by the wild-type and Bcl6-deficient Tregs was also evident by histological assessment of colon sections and following scoring in a blinded fashion (Figure 11C and 11D). Together, these data demonstrate that Bcl6-deficient Tregs are competent at suppressing Th1 inflammatory responses *in vivo* as wild-type Tregs.



**Figure 11: Bcl6-deficient Tregs are capable of suppressing Th1-mediated inflammatory responses *in vivo*.**

(A) Bcl6-deficient Foxp3-gfp Tregs (KO, black line) or their wild-type counterparts (WT, light grey line) were co-transferred i.p. along with Il10-deficient Tresps to separate cohorts of Rag1-deficient mice to assess reversal of disease. Data represents percent weight change relative to initial weight averaged for 3-4 mice per cohort and assessed over a 4-wk time period (\* $p < 0.05$  and \*\* $p < 0.01$  for cohorts receiving Tresps alone relative to cohorts receiving Tresps with either wild-type or Bcl6-deficient Tregs,  $p = \text{NS}$  between cohorts receiving wild-type and Bcl6-deficient Tregs).

(B) Severity of colitis assessed by changes in colon length 4 wks post-transfer to Rag1-deficient mice as described in (A).

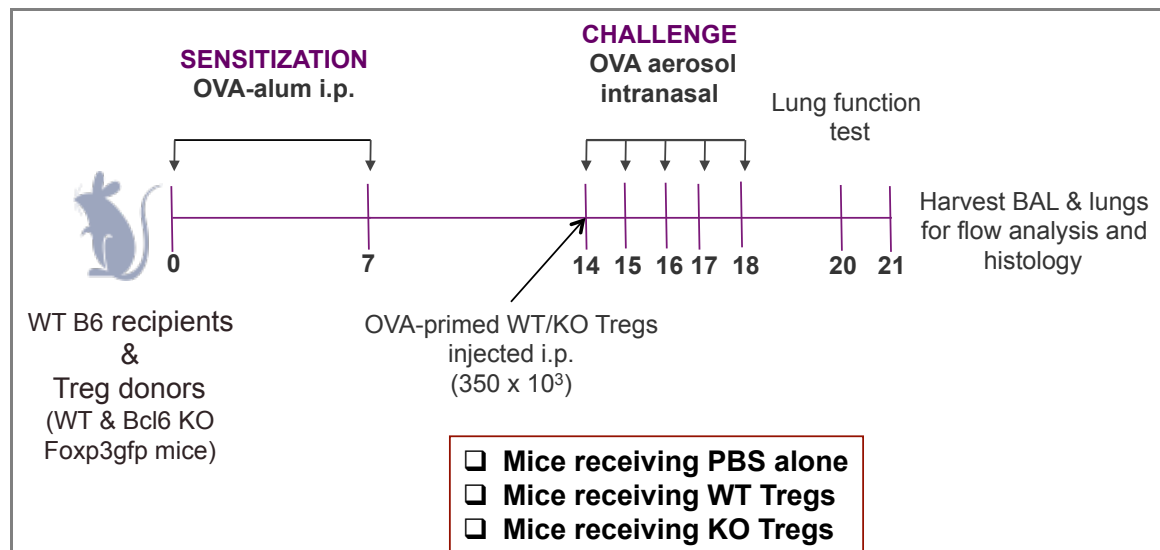
(C) Representative colon histopathology for the 3 cohorts of Rag1-deficient mice as described in (A) assessed by hematoxylin and eosin (H&E) staining 4 wks post-transfer (Original magnification - 200X for Rag1-deficient mice receiving Il10<sup>-/-</sup> Tresps alone and 400X for those receiving Il10-deficient Tresps plus either Bcl6-deficient or wild-type Tregs).

(D) Colon sections from the 3 cohorts of Rag1-deficient mice were scored 4 wks post-transfer in a blinded-fashion on a scale of 0-6.

(B,D) \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  (One-way analysis of variance) (error bars=s.e.m.)

## Bcl6-deficient Tregs promote rather than suppress Th2-driven allergic airway inflammation

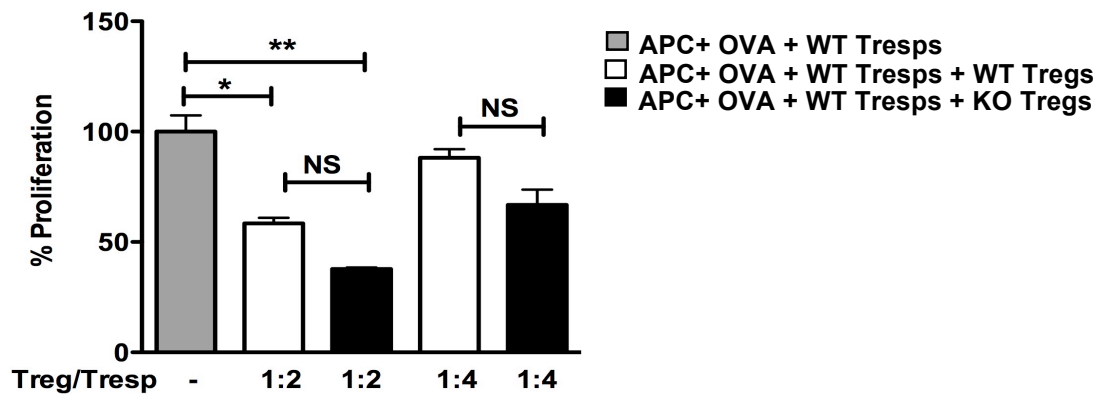
Since Bcl6-deficient mice develop spontaneous Th2-type inflammation, we next tested whether Bcl6-deficient Tregs were defective in their ability to suppress Th2 responses. We used a model where Th2 airway inflammation is induced by repeated intranasal administration of Ovalbumin (Ova) following initial i.p. priming with Ova (Chang *et al.*, 2010; Presser *et al.*, 2008) (Figure 12). Prior to the Ova challenge, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs from wild-type or Bcl6-deficient mice were injected to test their ability to suppress airway inflammation. Tregs were isolated from Ova primed mice, in order to obtain Ova-specific Tregs as shown (Figure 12). After the last Ova challenge, the mice were analyzed for airway hyper-reactivity by plethysmography (Figure 14).



**Figure 12: Schematic for testing Treg suppression of Th2 responses *in vivo* in an Ova-induced allergic airway inflammation model.**

Both the Treg donor (Bcl6-deficient, KO and wild-type, WT Foxp3gfp mice) and recipients (WT B6 female mice) were sensitized intra-peritoneally (i.p.) with Ova/Alum on days 0 and 7. On day 14, OVA-primed Tregs were then injected i.p. ( $350 \times 10^3$  cells/mouse) into the sensitized wild-type B6 female recipients. The recipient mice were then challenged intranasally with Ova for 5 consecutive days following which the mice were tested for lung function on day 20 and sacrificed for analysis of Th2 inflammation parameters.

We first tested the suppressor function of the OVA-primed Tregs from wild-type and Bcl6-deficient mice *in vitro* in the standard Treg suppression assay. Here, the isolated OVA-primed wild-type and Bcl6-deficient Tregs were co-cultured separately with wild-type Tresp cells activated in the presence of OVA-antigen. We noted that OVA-primed Bcl6-deficient Tregs were equally effective as wild-type Tregs in suppressing the proliferation of OVA-primed T cell responses *in vitro* (Figure 13). Thus, this indicated that Bcl6-deficient Tregs maintain their suppressor function after *in vivo* priming with OVA/Alum.

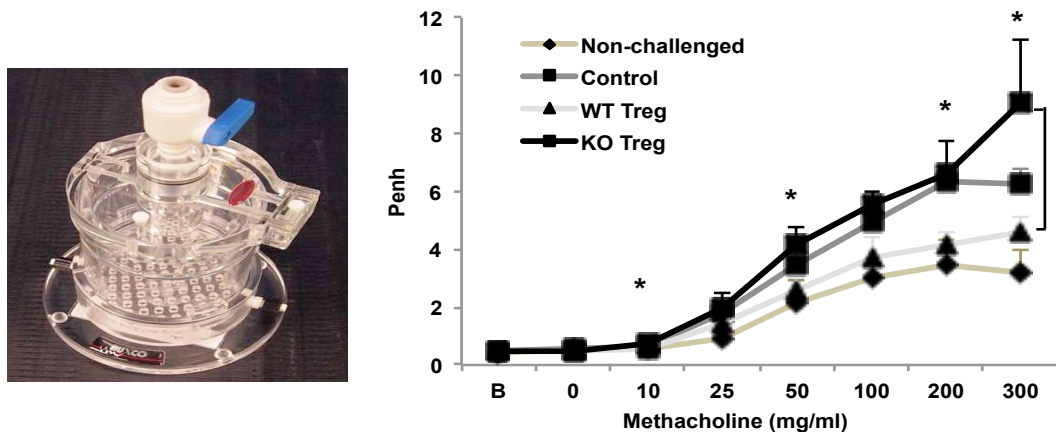


**Figure 13: Bcl6-deficient Tregs maintain intact suppressor function after priming with antigen.**

Suppression of Ova-primed T cell proliferative responses by CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs sorted from Bcl6-deficient (KO) (black bars) and wild-type (WT) (white bars) Foxp3-gfp mice sensitized with Ova following co-culture with wild-type CD25<sup>+</sup>Foxp3<sup>+</sup> (Tresp) cells (grey bar) activated with Ova (10 µg/ml) plus mitomycin-C treated APCs for 72 hrs (Treg/Tresp ratios - 1:2 and 1:4). Proliferation assessed as [<sup>3</sup>H] thymidine incorporation. Data plotted as percent proliferation is representative of two independent experiments (mean and s.e.m. of triplicate cultures). \*p< 0.05, \*\*p<0.01 (One-way analysis of variance).

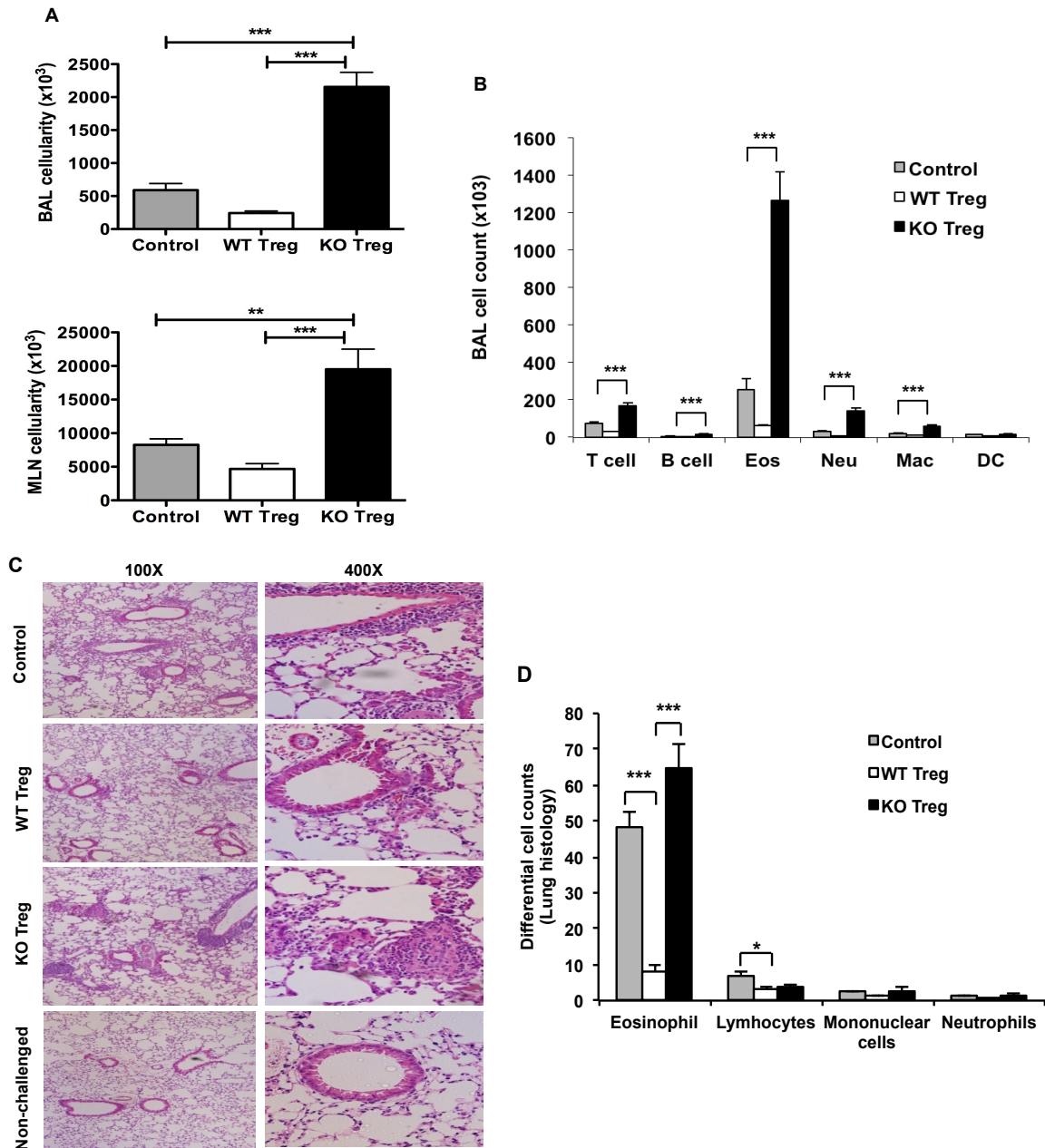


Allergic airway inflammation is associated with increased eosinophilic infiltration, pulmonary obstruction and airway hyper-responsiveness (Nials and Uddin, 2008). We therefore assessed lung function in the different cohorts of recipient mice subject to airway inflammation protocol by whole-body plethysmography (Figure 14). This entails exposure of the recipient mice to increasing doses of aerosolized methacholine and assessment of the enhanced pause response. As shown in Figure 14, wild-type Tregs suppressed the increased Penh response induced by methacholine, compared to control mice that did not receive Tregs. In contrast, mice receiving Bcl6-deficient Tregs displayed a similar Penh response as the control mice with inflammation, and at the highest dose of methacholine, showed a higher Penh reading than the control mice. These data indicate that Bcl6-deficient Tregs were not functioning to suppress airway responsiveness.



**Figure 14: Bcl6-deficient Tregs fail to control airway hyper-responsiveness *in vivo* in allergic airway inflammation model.** Assessment of lung function in Ova-sensitized and intranasally challenged wild-type recipient mice immunized i.p. with or without Bcl6-deficient (KO) or wild-type (WT) Tregs or left non-challenged (NC) (n = 5 per group). Airway hyper-reactivity was measured by the enhanced pause value by whole body plethysmography of the recipient mice 24 hrs after the final intranasal challenge, tested at baseline (B), and following inhalation of increasing doses of aerosolized methacholine (horizontal axis). \*p<0.05 (Student's t-test) between mice receiving Bcl6-deficient vs. wild-type Tregs.

To further analyze the airway inflammatory response, we measured the cellularity of the bronchoalveolar lavage (BAL) and of the draining mediastinal lymph nodes (MLN) for the three treatment groups. Strikingly, whereas wild-type Tregs suppressed BAL and MLN cell counts compared to control mice with inflammation, Bcl6-deficient Tregs promoted a several fold increase in BAL and MLN cellularity compared to control mice (Figure 15A). Analysis of the specific cell types in the BAL revealed that the bulk of the increase in cellularity was due to eosinophils, a cell type characteristic of Th2 mediated allergic airway responses (Figure 15B). This was also evident following histological examination of lung inflammation and blinded scoring of the inflammation by differential cell counts (Figure 15C and 15D). As shown in the figure, whereas wild-type Tregs clearly suppressed inflammatory cell accumulation around the airways, Bcl6-deficient Tregs failed to suppress the inflammation. These data indicate that Bcl6-deficient Tregs are not just severely defective in controlling inflammatory cell infiltration in the inflamed lungs, but that Bcl6-deficient Tregs actively exacerbate the lung inflammation, leading to greater recruitment of eosinophils and other inflammatory cell-types.



**Figure 15: Bcl6-deficient Tregs fail to control inflammatory cell accumulation *in vivo* in allergic airway inflammation model.**

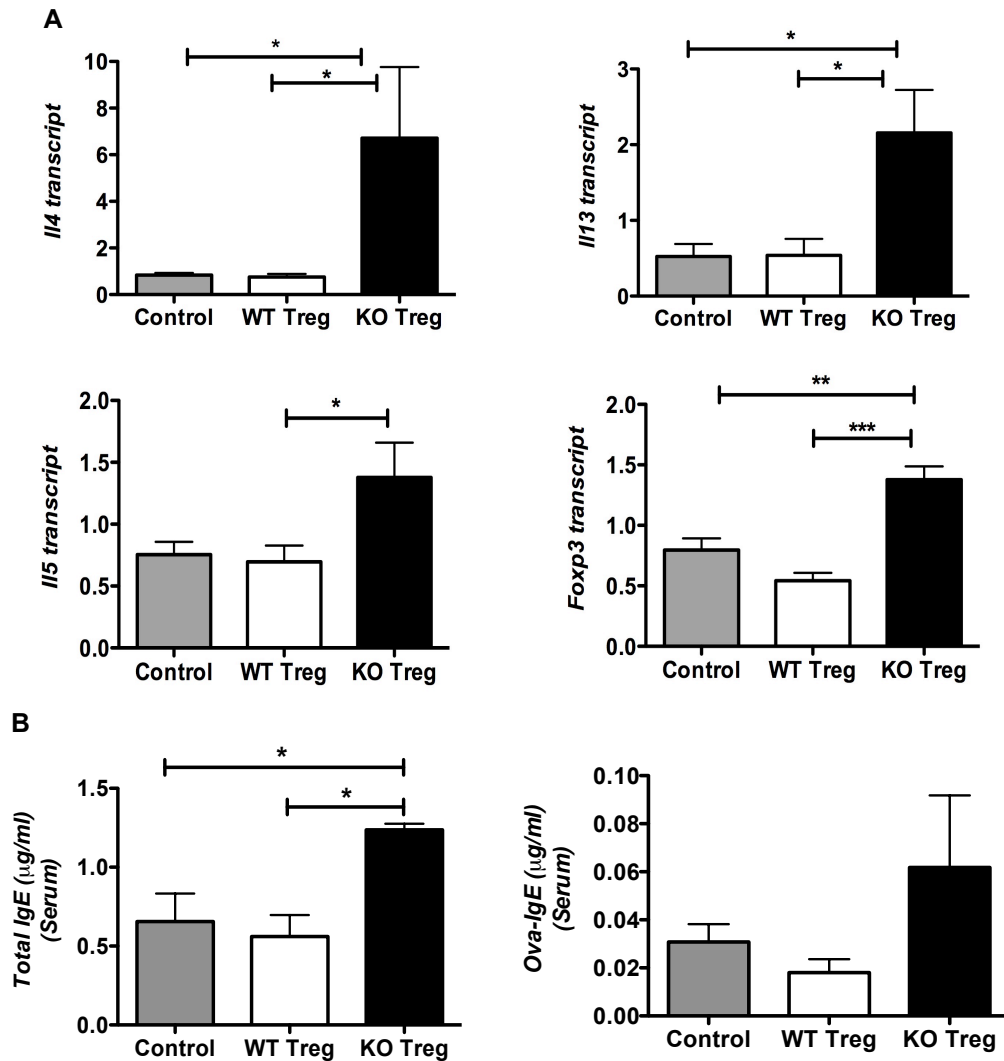
(A) Cellularity in the bronchoalveolar lavage (BAL) and the lung mediastinal lymph nodes (MLNs) for the Ova-sensitized and challenged recipient mice immunized with or without Bcl6-deficient (KO) or wild-type (WT) Tregs.

(B) Cellular composition of the BAL for the mice challenged as in (A) assessed by flow cytometry (Eos=Eosinophils, Neu=Neutrophils, Mac=Macrophages, DC=Dendritic cells).

(C) Lung histopathology assessed by H & E staining of paraffin-embedded lung sections for mice challenged as in (A) or left non-challenged (NC). Original magnification, 100 X (left) and 400 X (right).

(D) Differential cell counts of specific inflammatory cell types in 10 different fields of H & E-stained lung sections of the mice treated as in (A).

We next analyzed gene expression in the lungs by quantitative PCR analysis. We tested Foxp3 expression as a marker for Tregs, and found the highest Foxp3 expression in the lungs from mice injected with Bcl6-deficient Tregs (Figure 16A). This ruled out the possibility of defective homing of the Bcl6-deficient Tregs to the inflamed lungs. More importantly, we found that the Th2 cytokines *Il4*, *Il5* and *Il13* were most strongly expressed in the lungs of mice that received Bcl6-deficient Tregs (Figure 16A). The increased Th2 cytokines can account for the increased eosinophilic infiltration, impaired lung function and elevated levels of total IgE in the sera of mice receiving Bcl6-deficient Tregs than the other groups (Figure 16B). Overall, all these data demonstrated that Bcl6-deficient Tregs are severely defective in their ability to suppress the Th2 inflammatory responses *in vivo*. This selective failure to suppress Th2 responses can explain the severe Th2-mediated inflammatory disease that develops in Bcl6-deficient mice.



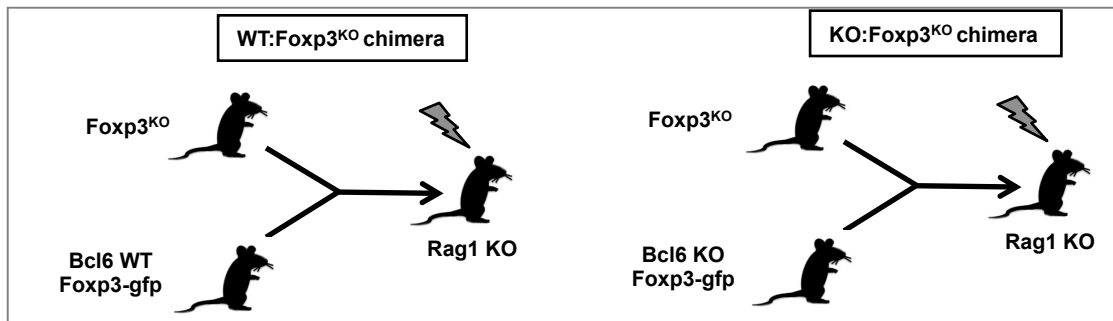
**Figure 16: Bcl6-deficient Tregs augment Th2 responses *in vivo* in allergic airway inflammation model.**

**(A)** Quantitative PCR analysis of Th2 cytokine genes (*Il4*, *Il5*, *Il13*) and *Foxp3* in the total lung RNA of Ova-sensitized and intranasally challenged wild-type recipient mice (grey bars) or immunized i.p. with Bcl6-deficient (KO) Tregs (black bars) or wild-type (WT) Tregs (white bars). Results are normalized to  $\beta_2$ -microglobulin.

**(B)** ELISA of total IgE and OVA-specific IgE in the serum of Ova-sensitized and intranasally challenged wild-type recipient mice (grey bars) or immunized i.p. with Bcl6-deficient Tregs (black bars) or wild-type Tregs (white bars).  $n = 5$  mice per group.

## Bcl6-deficient Tregs are selectively impaired in controlling systemic Th2 responses

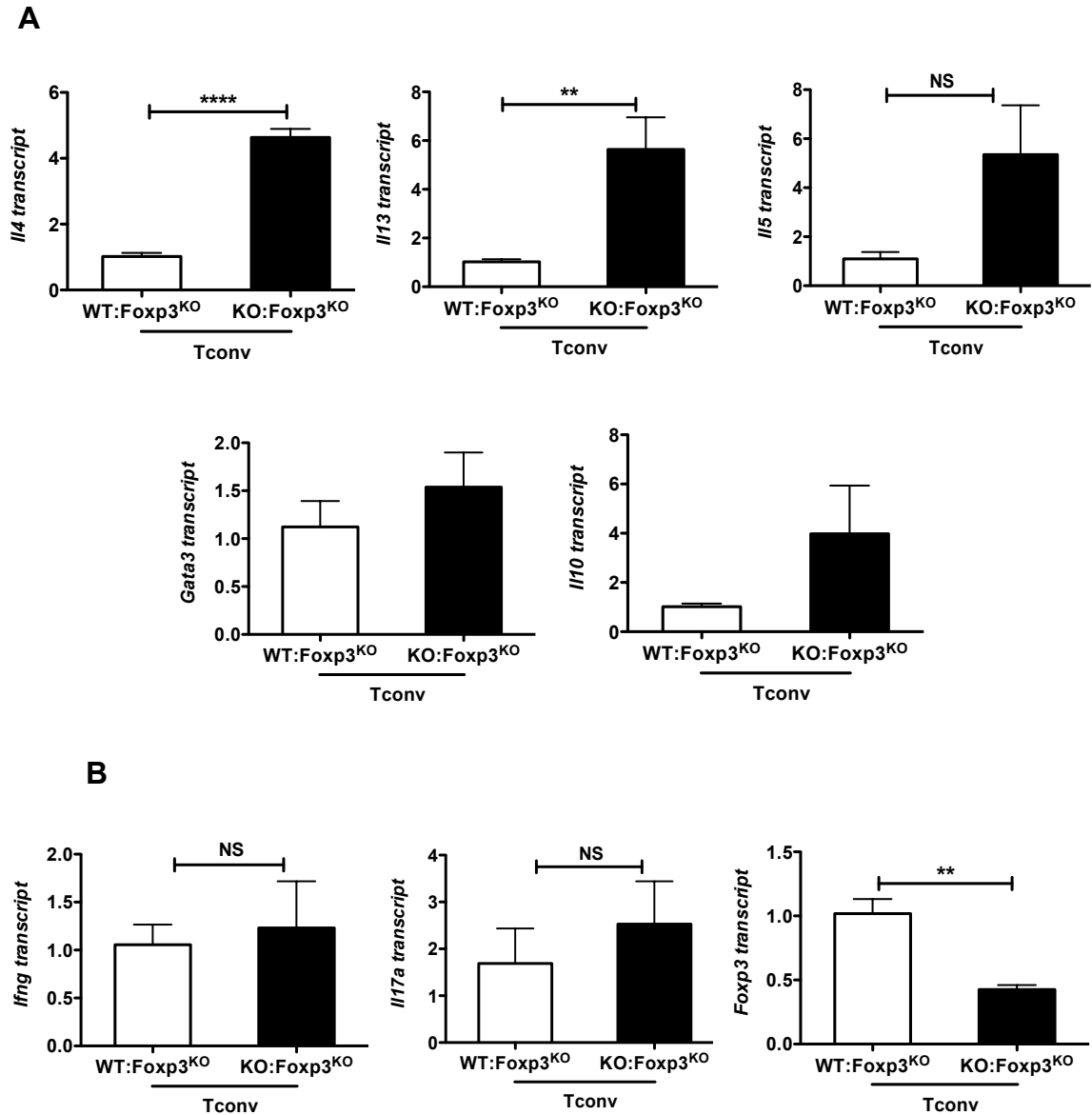
Since the Bcl6-deficient Tregs demonstrated a selective impairment in the control of Th2 responses in the airway challenge model, but retained intact function in the Th1 colitis model, we next tested their ability to control systemic inflammatory T cell responses in another *in vivo* setting. Foxp3-deficient or scurfy mice lack functional Tregs and develop a severe multi-organ autoimmune pathology that manifests as wasting disease, diabetes, pancreatitis, hyper-IgE, exfoliative dermatitis and cytokine storm. This fulminant autoimmunity is associated with exacerbated Th1 and Th2 responses in the absence of Tregs (Koch *et al.*, 2009; Liston *et al.*, 2008; Singh *et al.*, 2007). Therefore, taking advantage of this model to tease the functional competency of Bcl6-deficient Tregs to control Th1 vs. Th2 responses, we set up mixed bone marrow chimeras where bone marrow from Foxp3<sup>KO</sup> (*scurfy*) mice was injected into irradiated Rag1-deficient recipient mice along with bone marrow from wild-type Foxp3-gfp mice (WT:Foxp3<sup>KO</sup> chimeras) or Bcl6-deficient Foxp3-gfp mice (KO:Foxp3<sup>KO</sup> chimeras) (Figure 17).



**Figure 17: Schematic for setting up Foxp3<sup>KO</sup> BM chimeras.**

Scheme depicting the two types of bone marrow (BM) chimeras generated with Rag1 KO mice as recipients: wild-type (WT) BM mixed with Foxp3<sup>KO</sup> BM (WT:Foxp3<sup>KO</sup>) and Bcl6-deficient (KO) BM mixed with Foxp3<sup>KO</sup> BM (KO:Foxp3<sup>KO</sup>).

WT:Foxp3<sup>KO</sup> chimeric mice have a mixture of wild-type and Foxp3-deficient conventional T cells (Tconv cells) and a functional wild-type Treg subset, while KO:Foxp3<sup>KO</sup> chimeric mice have a mixture of Bcl6-deficient and Foxp3-deficient conventional T cells (Tconv cells) and complete deficiency of Bcl6 in the Treg subset. After 16-20 weeks of immune reconstitution, both types of chimeric mice were immunized with Ova-Alum to promote a Th2-type immune response, and Tconv cells were purified and tested for gene expression, following activation with anti-CD3 and anti-CD28 Abs *in vitro*. As shown in Figure 18A and 18B, Tconv cells from KO:Foxp3<sup>KO</sup> chimeras showed a striking increase in the expression of Th2 cytokines, compared to Tconv from WT: Foxp3<sup>KO</sup> chimeras, while *Ifng* and *Il17a* levels were not significantly different between the two chimera types. Also the Th2 transcription factor, *Gata3* was increased in Tconv cells from KO:Foxp3<sup>KO</sup> chimeras, relative to WT:Foxp3<sup>KO</sup> chimeras (Figure 18A). Thus, considering the abnormal development of Th1 and Th2 cells in Foxp3-deficient mice (Koch *et al.*, 2009; Liston *et al.*, 2008; Singh *et al.*, 2007), the specific increase in Th2 cytokines but not Th1 cytokines with the KO:Foxp3<sup>KO</sup> chimeras further confirms the selective failure of Bcl6-deficient Tregs to control Th2 responses but not Th1 and Th17 responses. Thus, our results with the KO:Foxp3<sup>KO</sup> chimeras and the Th1 and Th2 disease models clearly demonstrate a critical role for Bcl6 in Treg-mediated control of Th2 inflammatory responses *in vivo*.



**Figure 18: Bcl6-deficient Tregs are selectively impaired in the control of systemic Th2 inflammatory responses *in vivo*.**

Quantitative PCR analysis of Th2 genes (*Il4*, *Il13*, *Il5*, *Gata3*, *Il10*) (**A**) and (*Ifng*, *Il17a*, *Foxp3*) (**B**) in Foxp3<sup>KO</sup> bone marrow chimera-derived wild-type (WT) and Bcl6-deficient (KO) Tconv cells with expression normalized to *tubb5*. Data show average expression from 4 mice for (WT: Foxp3<sup>KO</sup>) group and 3 mice for (KO:Foxp3<sup>KO</sup>) group. \*\*p<0.01, \*\*\*\*p<0.0001 (Student's t-test) (error bars=s.e.m.)



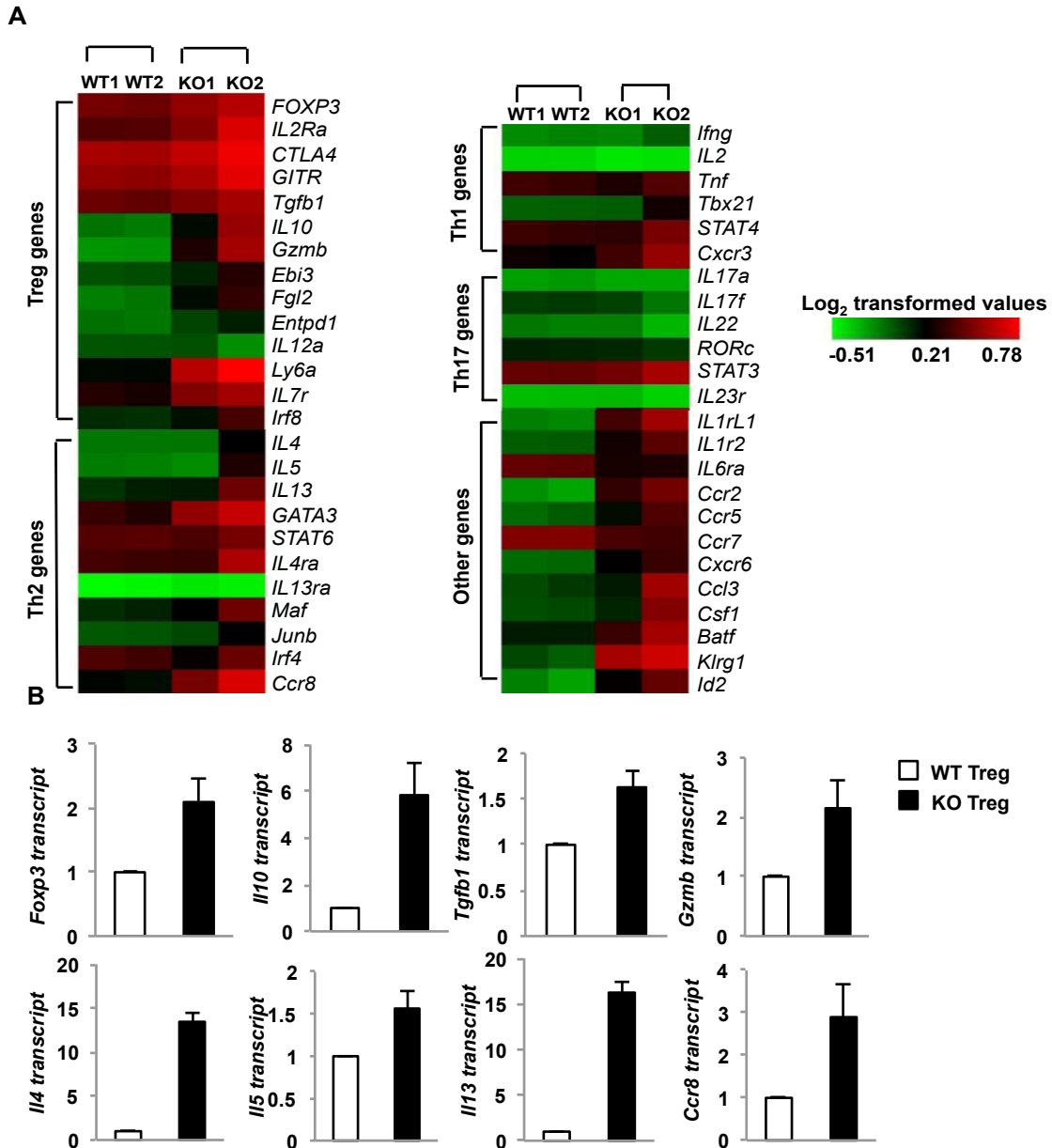
### **Bcl6 represses the expression of both Treg and Th2 genes in Treg lineage**

Next, to understand the functional defect that can explain the selective failure of Bcl6-deficient Treg cells to control Th2 immune responses, we performed a gene expression microarray using mRNA from highly purified CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs from wild-type and Bcl6-deficient mice, activated *in vitro* with anti-CD3 and anti-CD28 Abs. We found a large number of gene differences between wild-type and Bcl6-deficient Tregs, with 833 genes being up-regulated more than 1.5-fold in Bcl6-deficient Treg cells in a statistically significant manner. Several of the hallmark Treg suppressor genes, such as *Foxp3*, *Il2ra*, *Ctla4* and *Tgfβ1* were up-regulated in Bcl6-deficient Tregs, relative to their wild-type counterparts (Figure 19A, 19B). *Il10* and Granzyme B (*Gzmb*), two important components of the Treg genetic program were strikingly up-regulated in Bcl6-deficient Tregs (Figure 19A). Both these genes have previously been reported as Bcl6 targets in T cells (Kusam *et al.*, 2003; Yoshida *et al.*, 2006), confirming that Bcl6 is a critical repressor of gene expression in Treg cells. Many of these Treg suppressor genes are also increased in CD4<sup>+</sup>CD25<sup>hi</sup> Bcl6-deficient Tregs (Chung *et al.*, 2011), however we have noted that this Treg population can also include activated CD25<sup>hi</sup>Foxp3<sup>-</sup> Th2 effectors. Therefore, our microarray analysis with CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs identifies direct targets of Bcl6 in Tregs. The up-regulation of Treg suppressor genes in Bcl6-deficient Treg cells can explain the enhanced ability of Bcl6-deficient Tregs to suppress T cell proliferation *in vitro*. Also, the significant up-regulation of *Il10* and *Tgfβ1* can explain their ability to effectively maintain gut homeostasis in the Th1-mediated colitis model.

Interestingly, in addition to the increased Treg suppressor genes, Bcl6-deficient Tregs also displayed increased expression of multiple Th2 lineage genes (Figure 19A, 19B), most notably the Th2 transcription factors (*Gata3*, *Maf*), Th2 cytokines (*Il4*, *Il5* and *Il13*) as well as Th2-associated chemokine receptors like *Ccr8*. The increased Treg and Th2 gene expression of Bcl6-deficient Tregs indicated that Bcl6-deficient Tregs acquire a hybrid Treg-Th2 phenotype and this may contribute to their failure in control of Th2 immune responses. Thus, Bcl6 may be essential for repression of Treg and Th2 gene expression in Tregs. *Irf4*, another Th2-associated transcription factor has been demonstrated to be critical for Treg control of Th2 inflammatory responses (Zheng *et al.*, 2009). However, the expression of *Irf4* was not significantly different between wild-type and Bcl6-deficient Tregs, suggesting that Bcl6 regulates Th2 gene expression in Tregs independent of *Irf4*. Importantly, Th1 and Th17 lineage genes, were not significantly different between wild-type and Bcl6-deficient Tregs (Figure 19A, 19B), further highlighting the specific de-regulation of the Th2 gene expression program in Tregs in the absence of Bcl6. In addition, several chemokine receptor genes were up-regulated in the Bcl6-deficient Tregs, as well as other genes with important immune system functions such as *Csf1*, *Batf*, *Il1rl1* and *Klrg1*. These data indicate that Bcl6 expression in Tregs is also critical for limiting the effector profile of Treg cells.

In the microarray, “KO2” showed a much higher expression of both Treg genes and Th2 genes than “KO1”, consistent with KO2 having a greater severity of Th2 inflammation than KO1 as assessed by histology of the heart and lungs

(D.V.S., A.L.D., data not shown). Th1 and Th17 genes were also modestly up-regulated in KO2, but not KO1. This increase can be explained by the reported role for Bcl6 in inhibiting Th1 and Th17 differentiation (Nurieva *et al.*, 2009; Yu *et al.*, 2009). However, the role of Bcl6 in inhibiting Th2 responses is more dominant for the inflammatory phenotype of Bcl6-deficient mice, as Tregs from both the healthy and sick Bcl6-deficient mice show an increase in Th2 gene expression. This indicates that, in addition to an intrinsic role for Bcl6 in regulating Th2 gene expression in Tregs (as shown by healthier KO1), the Th2 inflammatory disease further contributes to “remodeling” of the Treg cells (as shown by sicker KO2) and both these effects underlie the defective control of Th2 responses by Bcl6-deficient Tregs.



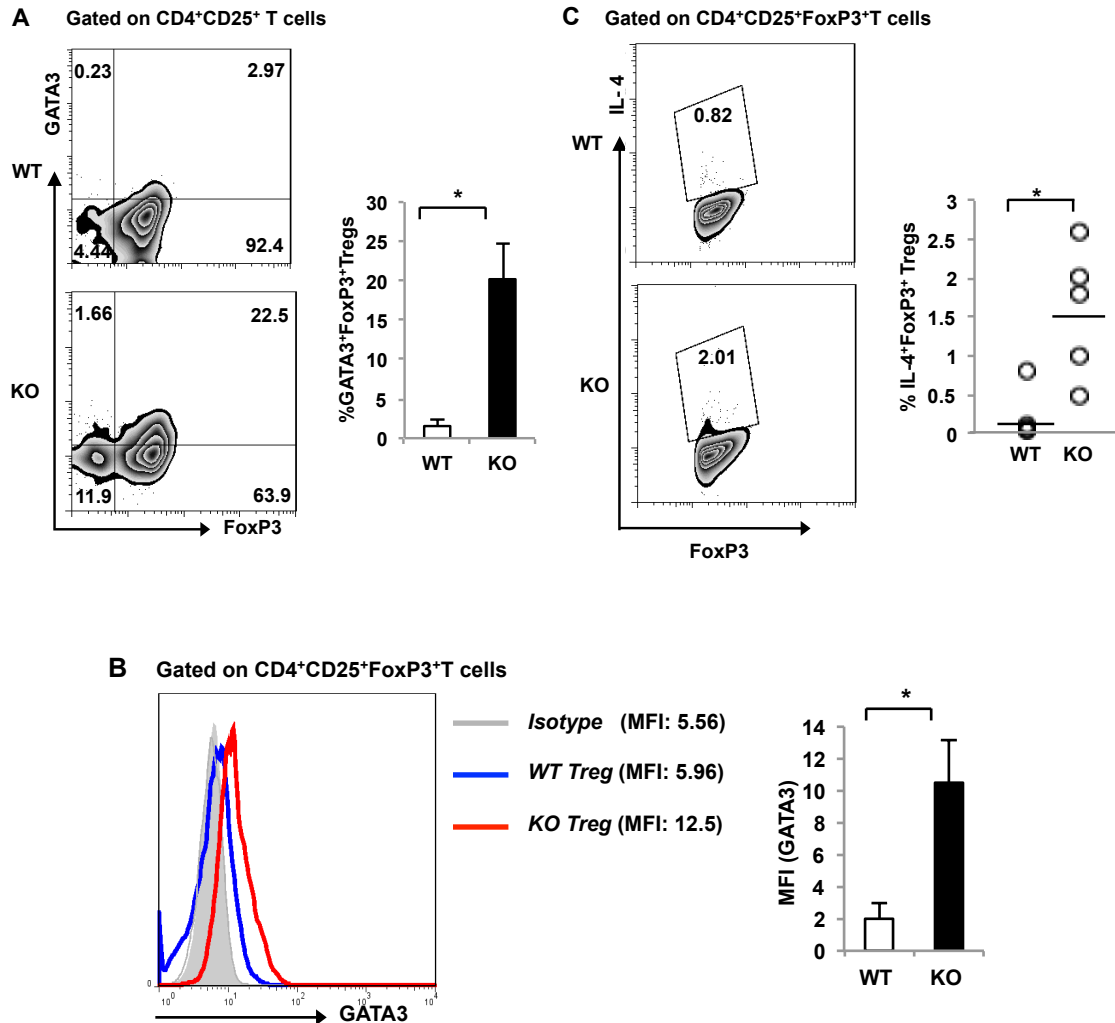
**Figure 19: Bcl6-deficient Tregs exhibit a hybrid Treg-Th2 phenotype.**

(A) Heat map depiction of gene transcripts differentially expressed with statistical significance between sorted Tregs from Bcl6-deficient (KO) or wild-type (WT) Foxp3-gfp mice, analyzed by expression microarrays. Gene expression represented as Log<sub>2</sub> transformed values with red color representing expression greater than mean and green color representing expression less than the mean as shown in the color scale on the right (n= 2 mice per group, with 2 replicates each mouse represented as an average, KO1 – healthy, KO2 - sick).

(B) Quantitative PCR validation of differentially expressed genes between Bcl6-deficient (KO) Tregs (black bars) and wild-type (WT) Tregs (white bars) selected from the microarray analysis following activation for 16 hrs with anti-CD3 and anti-CD28 (Treg genes - *Foxp3*, *Il10*, *Tgfb1* and *Gzmb*) and Th2 genes (*Il4*, *Il5*, *Il13* and *Ccr8*), with expression normalized to  $\beta$ -tub. Data are average expression from at least 3 different mice per group.

## **Bcl6 represses Th2 genes in Tregs, independent of Foxp3 expression**

Treg cells can down-regulate expression of Foxp3 in inflammatory environments and gain the potential to produce effector cytokines (IFN $\gamma$  and IL-17) (Pasare and Medzhitov, 2003; Wan and Flavell, 2007; Zhou *et al.*, 2009c). Studies using elegant dual reporter mice to monitor Foxp3 expression and Treg stability have identified a small population of “exTregs” (Tregs that had expressed Foxp3 at an earlier time-point) that expand in autoimmune settings and secrete pro-inflammatory cytokines (Zhou *et al.*, 2009c). Therefore, in order to confirm if the increased Th2 genes noted by microarray analysis was not due to any contaminating Th2 effectors, we stained Bcl6-deficient Tregs for Foxp3 and Th2 genes. The percentage of Gata3<sup>+</sup> T cells was higher in Foxp3<sup>-</sup> Tconv cells in Bcl6-deficient mice relative to wild-type (Figure 20), consistent with the established role for Bcl6 in repressing Gata3 expression in T cells (Kusam *et al.*, 2003). Treg cells are reported to exhibit an intrinsic Th2 bias (Wang *et al.*, 2010), with increased Gata3 expression in *ex vivo* isolated wild-type Tregs than Tconv cells (Wang *et al.*, 2011; Wohlfert *et al.*, 2011). Importantly, we noted increased percentages of Foxp3<sup>+</sup>Gata3<sup>+</sup> Tregs as well as elevated Gata3 expression in the Foxp3<sup>+</sup> Tregs in Bcl6-deficient mice (Figure 20A, 20B). Correspondingly, there was also a significant increase in the percentage of IL-4<sup>+</sup>Foxp3<sup>+</sup> Tregs in Bcl6-deficient mice relative to wild-type (Figure 20C). Thus, in agreement with the gene expression array data, we noted increased Th2 gene expression in the Foxp3<sup>+</sup> Tregs in Bcl6-deficient mice. Thus, Bcl6-deficient Tregs acquired a novel hybrid Treg-Th2 phenotype, that could contribute to defective Th2 regulation.



**Figure 20: Bcl6 represses Th2 genes in Tregs, independent of Foxp3 expression.**

(A) (Left) Representative FACS plot depicting expression of Gata3 in freshly isolated Foxp3<sup>+</sup> and Foxp3<sup>-</sup> CD4<sup>+</sup>CD25<sup>+</sup> fractions from Bcl6-deficient (KO) and wild-type (WT) mice. (Right) Bar graph representing % Gata3<sup>+</sup>Foxp3<sup>+</sup> Treg cells in Bcl6-deficient (black bar) and wild-type (white bar) mice (n = 3 per group).

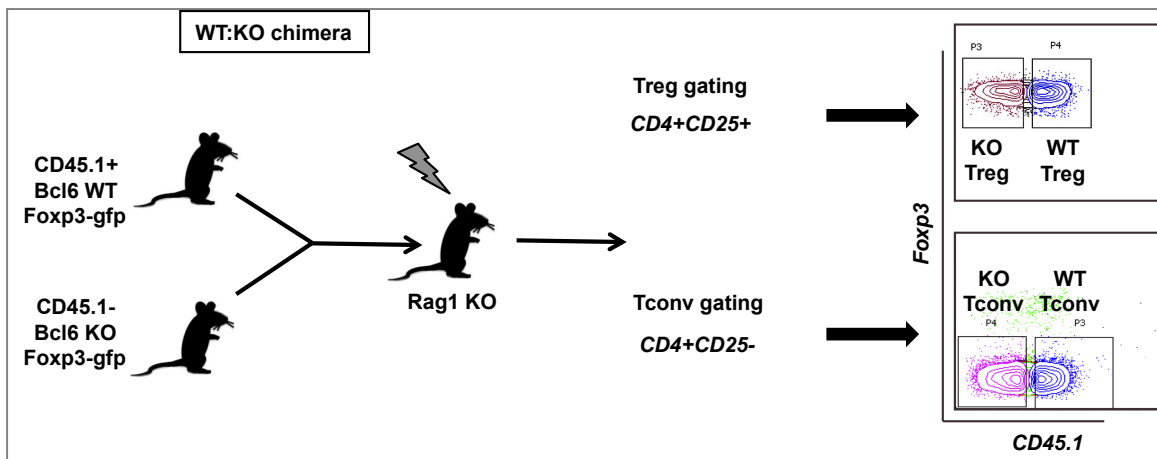
(B) (Left) Representative histogram depicting expression of Gata3 on freshly isolated Bcl6-deficient and wild-type cells gated on CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells. (Right) Mean fluorescence intensity (MFI) for Gata3 staining in the Bcl6-deficient (black bar) and wild-type (white bar) Foxp3<sup>+</sup> fraction is quantified (n = 3 per group).

(C) (Left) Representative FACS plot depicting expression of IL-4 on Bcl6-deficient and wild-type CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> fraction following stimulation with PMA and Ionomycin (Right) Scatter plot represents % IL-4<sup>+</sup>Foxp3<sup>+</sup> Treg cells in Bcl6-deficient and wild-type mice (n = 5 per group).

\*p<0.05 (Student's t-test) (error bars=s.e.m.)

## Th2 genes are intrinsically repressed by Bcl6 in Tregs

The critical next step was to definitively address if the up-regulated Th2 genes in Bcl6-deficient Tregs was due to intrinsic regulation by Bcl6 in the Treg lineage, or from an indirect effect from the ongoing Th2 inflammatory disease in the Bcl6-deficient mice. To test this, we generated mixed bone marrow chimeras where bone marrow from CD45.1<sup>+</sup> wild-type Foxp3-gfp mice was injected into sub-lethally irradiated Rag1-deficient recipient mice along with bone marrow from CD45.1<sup>-</sup> Bcl6-deficient Foxp3-gfp mice (WT:KO chimeras) (Figure 21). After 16-20 weeks of immune reconstitution, chimeric mice were immunized with Ova-Alum to promote a Th2-type immune response, and CD45.1<sup>+</sup> wild-type Foxp3-gfp and CD45.1<sup>-</sup> Bcl6-deficient Foxp3-gfp Tregs and Tconv cells were purified as shown in Figure 21 and tested for gene expression following activation *in vitro*.

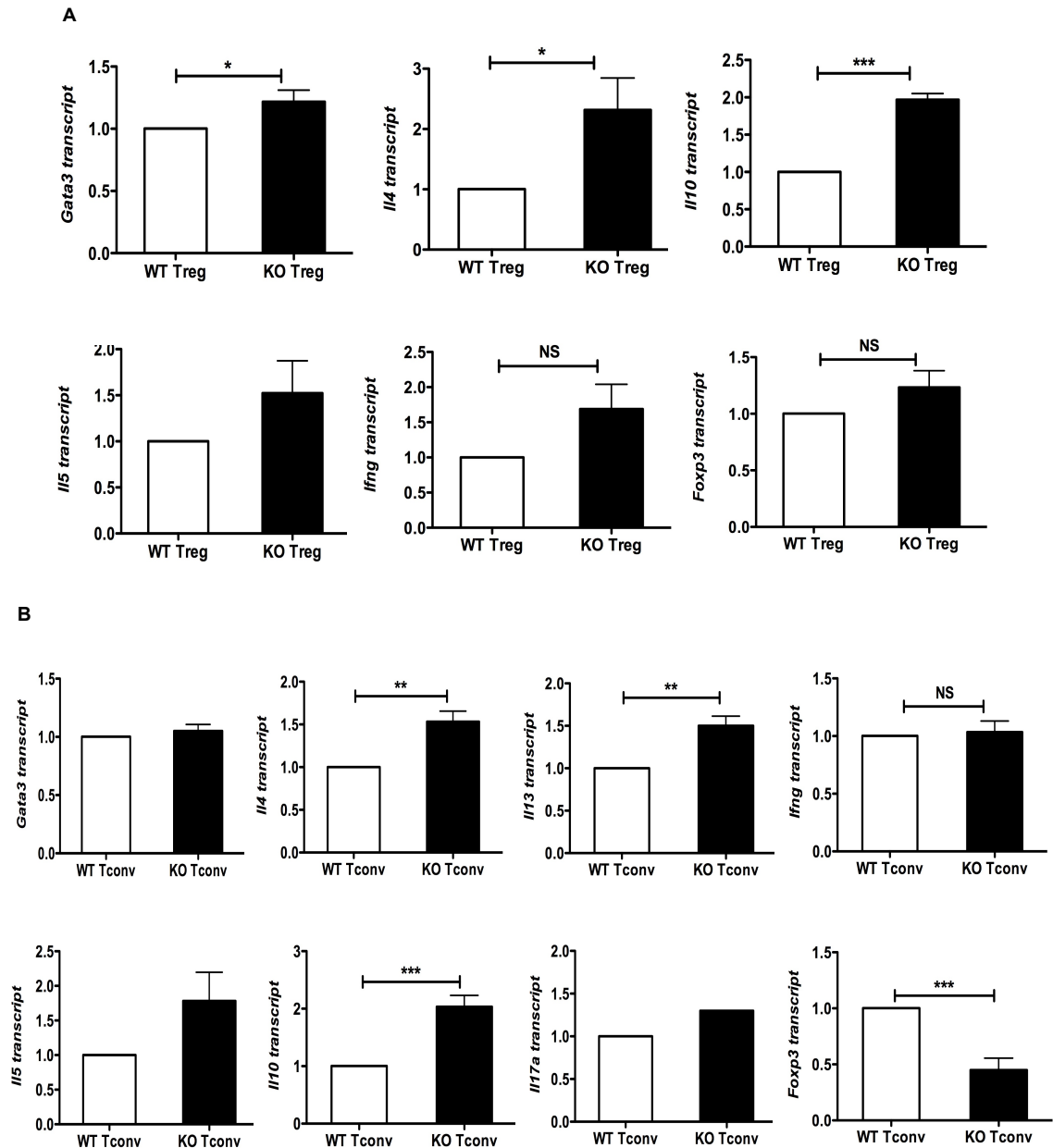


**Figure 21: Schematic for setting up WT:KO BM chimeras.**

Representative FACS sorting scheme for isolating Bcl6-deficient (KO) (CD45.1<sup>-</sup>) and wild-type (WT) (CD45.1<sup>+</sup>) Tregs and Tconv cells from mixed bone marrow chimeras; with Tregs gated as CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> (GFP<sup>+</sup>) T cells and Tconv cells gated as CD4<sup>+</sup>CD25<sup>-</sup> Foxp3<sup>-</sup> (GFP<sup>-</sup>) T cells, prior to gating based on CD45.1 expression.

As shown in Figure 22A and 22B, Gata3 expression was increased specifically in Bcl6-deficient Treg cells but not Bcl6-deficient Tconv cells. Since Gata3 can auto-activate its own expression (Ouyang *et al.*, 2000), an intrinsic increase of Gata3 in chimeric Bcl6-deficient Treg cells is consistent with the augmented Gata3 expression noted in Treg cells from Bcl6-deficient mice as shown in Figures 19 and 20. *Il4* and *Il10* were up-regulated 2-fold in Bcl6-deficient Tregs from the chimera compared to wild-type Tregs in the same chimera (Figure 22A), indicating that these genes are intrinsically repressed by Bcl6 in Tregs. We also observed increased intrinsic expression of *Il4*, *Il13* and *Il10* in Bcl6-deficient conventional T (Tconv) cells from the chimeric mice relative to wild-type Tconv cells, validating our previous work demonstrating Bcl6 as an inhibitor of Th2 responses (Kusam *et al.*, 2003) (Figure 22B). Overall, the up-regulation of Th2 genes in the Bcl6-deficient Treg and Tconv cells derived from mixed bone marrow chimeras was less dramatic compared to Th2 responses noted in germline Bcl6-deficient mice. This is potentially due to the presence of functional wild-type Treg counterparts in the chimeras that reduce the severity of overall Th2 inflammation. Indeed, Bcl6-deficient Tregs derived from KO:Foxp3KO chimeras showed several fold increase in Th2 genes, similar to germline Bcl6-deficient mice, since these chimeras lack functional wild-type Tregs due to Foxp3 deficiency (Figure 23). Nevertheless, the significant up-regulation of Th2 genes in the Bcl6-deficient Tregs in the chimeras strongly support an intrinsic role for Bcl6 in repressing Th2 gene expression in Tregs and their conversion to pro-inflammatory Th2 effector-like Tregs.

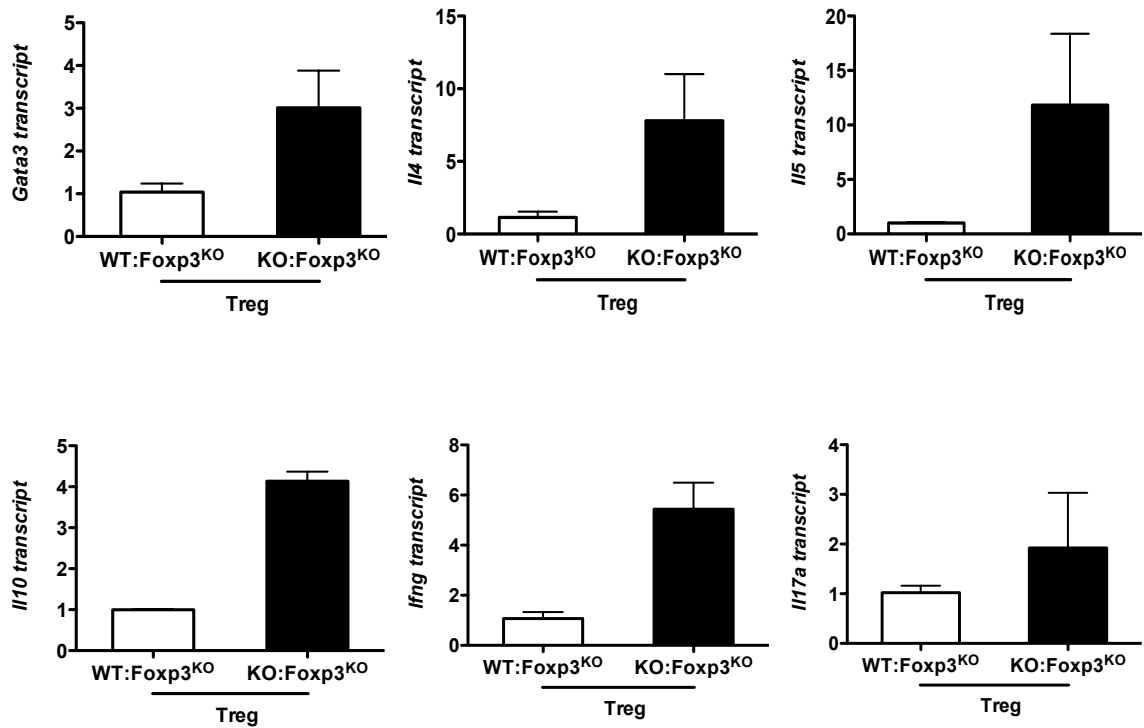




**Figure 22: Bcl6 intrinsically represses Th2 genes in Tregs.**

**(A)** Quantitative PCR analysis of *Gata3*, *Il4*, *Il10*, *Il5*, *Ifng* and *Foxp3* in sorted bone marrow chimera-derived Bcl6-deficient (KO) (CD45.1<sup>-</sup>) and wild-type (WT) (CD45.1<sup>+</sup>) Tregs with expression normalized to *tubb5*. Data show average expression from 6 different mice per group. \*p<0.05, \*\*\*p<0.001 (Student's t-test) (error bars=s.e.m.)

**(B)** Quantitative PCR analysis of transcripts for *Gata3*, *Il4*, *Il13*, *Ifng*, *Il5*, *Il10*, *Il17a* and *Foxp3* in sorted bone marrow chimera-derived Bcl6-deficient (KO) Tconv cells (CD45.1<sup>-</sup>) and wild-type (WT) Tconv counterparts (CD45.1<sup>+</sup>) with expression normalized to *tubb5*. Data show average of 6 different mice per group. \*\*p<0.01, \*\*\*p<0.001 (Student's t-test) (error bars=s.e.m.)

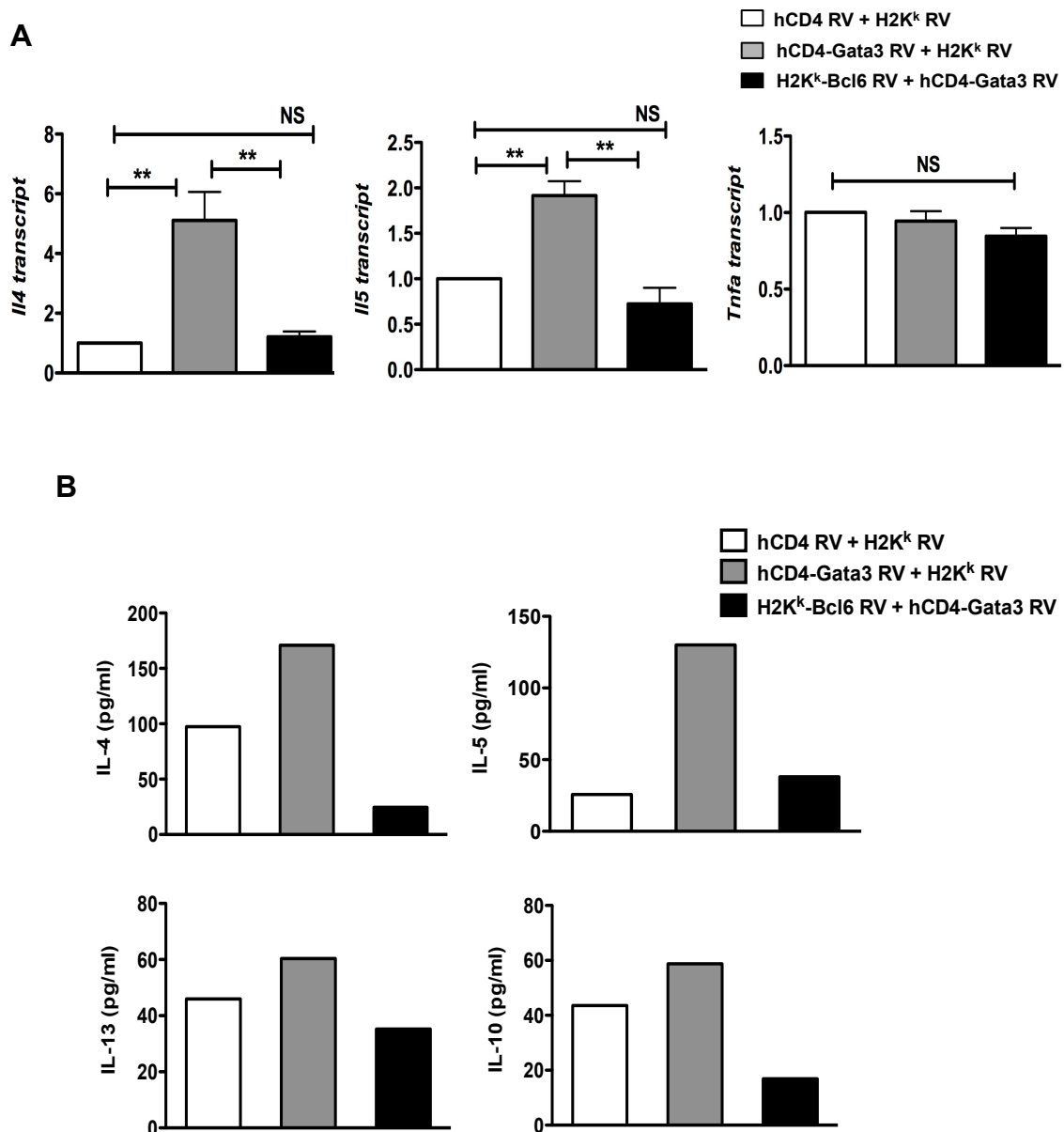


**Figure 23: Greater Th2 remodeling of Bcl6-deficient Tregs in KO:Foxp3<sup>KO</sup> chimeras than WT:KO chimeras.**

Quantitative PCR analysis of *Gata3*, *Il4*, *Il5*, *Il10*, *Ifng* and *Il17a* in Foxp3<sup>KO</sup> bone marrow chimera-derived wild-type (WT) and Bcl6-deficient (KO) Treg cells with expression normalized to *tubb5*. Data show average expression from 4 mice for (WT:Foxp3<sup>KO</sup>) group and 3 mice for (KO:Foxp3<sup>KO</sup>) group.

### **Bcl6 represses transcriptional activity of Gata3**

Bcl6 is well-established as an inhibitor of Th2 cell differentiation and Th2-type inflammation. This Th2 inhibition by Bcl6 is IL-4/STAT6 independent and has been demonstrated to involve post-transcriptional repression of Gata3 protein expression, although the exact mechanism is not yet clear (Dent *et al.*, 1998a; Kusam *et al.*, 2003). However, whether Bcl6 can also block the transactivation of Th2 cytokines by Gata3 is not yet explored. Increased *Gata3* expression as well as augmented Gata3 transcriptional activity in the absence of Bcl6 can explain the Th2-dominated phenotype of Bcl6-deficient mice. To address this, we tested the effect of Bcl6 on Gata3 transcriptional activity in two different functional assays. First, we induced Th2 cytokine expression in Stat6-deficient T cells by infecting with a Gata3-expressing retrovirus and tested the ability of Bcl6 to repress the Gata3-induced Th2 cytokine expression. As shown in Figures 24A and 24B, while Gata3 potently induced Th2 cytokine expression (both transcript and protein), Bcl6 essentially ablated the induction of IL4 and IL5 by Gata3, showing that Bcl6 could strongly repress Gata3 function in T cells. However, Bcl6 did not repress *Tnfa*, indicating that Bcl6 does not globally repress cytokine gene expression.

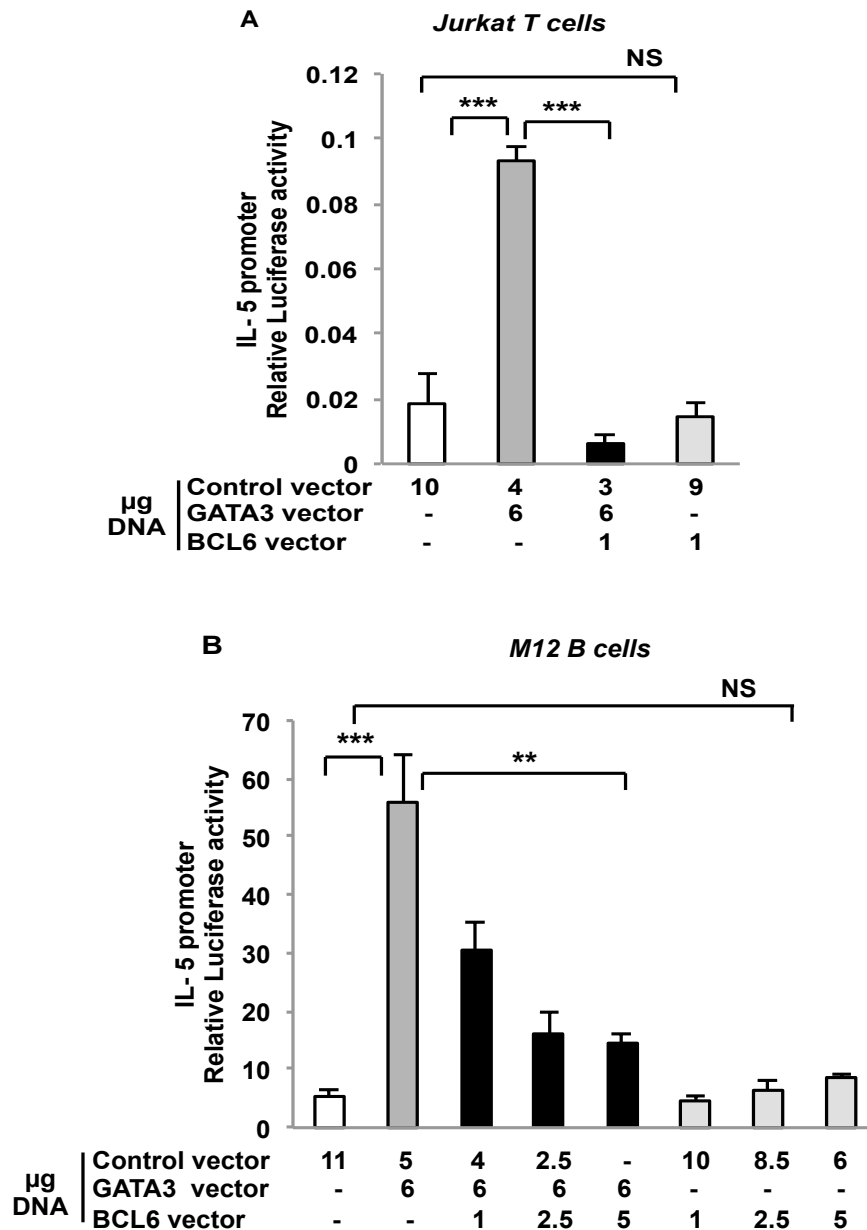


**Figure 24: Bcl6 represses Th2 cytokine-inducing ability of Gata3.**

**(A)** Quantitative PCR analysis of relative cytokine gene expression (*Il4*, *Il5*, *Tnfa*) for Stat6-deficient T cells retrovirally transduced with control RV, hCD4-Gata3 RV alone and double transduction of hCD4-Gata3 RV and H2K<sup>k</sup>-Bcl6 RV. Transduced cells were sorted using RV markers - hCD4 and H2K<sup>k</sup> and then re-stimulated with anti-CD3 and anti-CD28 for 4 hrs for analysis of gene expression. Expression normalized to  $\beta$ -*tub*. \*\* $p < 0.01$  (One-way analysis of variance)

**(B)** Levels of Th2 cytokines (IL-4, IL-5, IL-13 and IL-10) in samples treated as in (A) quantified by multiplex analysis.

As a second approach to further examine the repression of Gata3 function by Bcl6, we utilized a transient transfection assay in Jurkat T cells wherein luciferase expression is under the control of the *IL5* promoter. As shown in Figure 25A, Gata3 alone strongly activates the *IL5* promoter, and Bcl6 alone weakly represses *IL5* promoter activity. When co-transfected together, Bcl6 potently represses Gata3-induced *IL5* promoter activity, showing that Bcl6 can directly target and inhibit the transcriptional activity of Gata3. Similar repressive effects were also noted in M12 B cells (Figure 25B), thus showing that Bcl6 inhibits Gata3 function independent of endogenous Gata3 and does not require other T cell specific factors. Both these approaches demonstrate the critical role of Bcl6 in repressing Gata3 transcriptional function. Thus, the increased levels of Gata3 and increased Gata3 transcriptional activity in the absence of Bcl6 would lead to increased auto-activation of Gata3 (Ouyang *et al.*, 2000), thus amplifying the effects of loss of Bcl6, and strengthening Th2 lineage gene expression in Bcl6-deficient T cells.



**Figure 25: Bcl6 represses trans-activation of Th2 cytokines by Gata3.** Luciferase activity in Jurkat T cells (**A**) and M12 B cells (**B**) co-transfected with IL-5 promoter driven luciferase reporter and expression constructs for CXN, CXN-Gata3, CXN-Bcl6 and CXN-Gata3 plus CXN-Bcl6. Following electroporation, cells were stimulated with PMA and Ionomycin for Jurkat T cells or PMA plus dibutyryl cAMP 24 hrs prior to harvest and luciferase measurement. Results representative of 5 and 2 independent experiments, respectively. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  (Student's t-test) (error bars= s.e.m.).

## **Repression of Gata3 function by Bcl6 is critical in the Treg lineage**

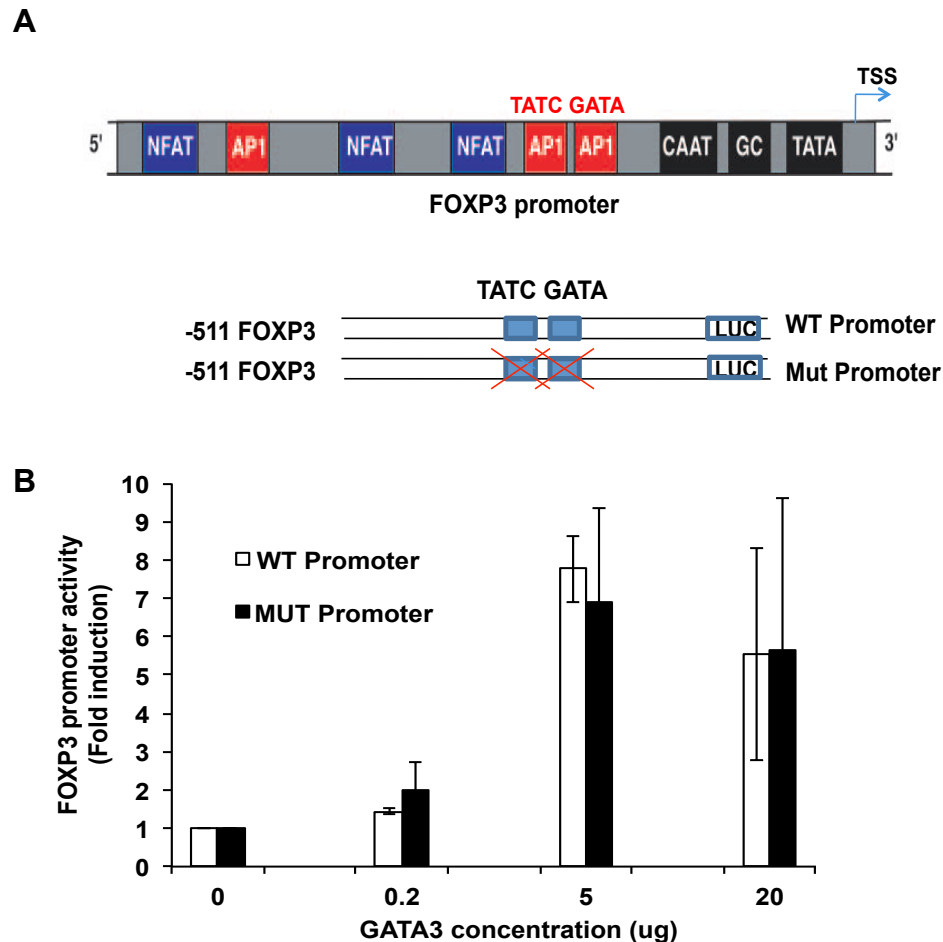
We next wanted to understand if the regulation of Gata3 transcriptional activity by Bcl6 is functional in the Treg lineage and how it relates to the Th2-bias of Bcl6-deficient Tregs and their failure to control Th2 responses. Gata3 and Th2 cytokines are known to antagonistically regulate Treg fate determination. At a mechanistic level, several lines of evidence support this long-standing premise in the field. IL-4 induces Stat6 that can bind to a silencer element in the Foxp3 promoter and repress TGF $\beta$ -induced Treg differentiation (Takaki *et al.*, 2008). IL-4 can also function by Gata3 induction via the canonical Stat6 signaling pathway, and enforced expression of Gata3 is sufficient to block Foxp3 induction (Wei *et al.*, 2007). Gata3 can directly bind to palindromic Gata site (located ~303 bp upstream of TSS) in the Foxp3 promoter and inhibit Foxp3 expression in Th2 cells (Mantel *et al.*, 2007). Foxp3 promoter activity was reported as significantly reduced in Jurkat T cells that constitutively express Gata3 (Mantel *et al.*, 2007). We however noted strikingly opposite results in performing similar tests to assess Gata3 regulation of Foxp3. We generated two different Foxp3 promoter constructs – wild-type promoter with the palindromic Gata site intact and a mutant promoter with both the Gata sites deleted by site-directed mutagenesis (Figure 26A). Both these promoter constructs were functional in Jurkat T cells. In contrast to the results in the Mantel study, we noted that co-transfection of Gata3 activated Foxp3 promoter activity, independent of the palindromic Gata site (Figure 26B). In agreement with our data, two recent studies have identified an indispensable role for Gata3 in the Treg lineage (Wang *et al.*, 2011; Wohlfert *et*

*et al.*, 2011). This is mainly attributed to a positive role for Gata3 in inducing Foxp3 expression and thus maintaining the hallmark Treg gene signature and functional fitness. In this study, Gata3 was shown to bind CNS2 element in the Foxp3 locus, while minimal binding was detected at the promoter, CNS1 and CNS3 elements in Treg cells. Reporter assays in Jurkat T cells identified that Gata3 positively enhanced Foxp3 promoter activity, although the effect was significantly stronger for promoter plus CNS2 construct. CNS2 is important for maintenance of Foxp3 expression in thymic Tregs, thus suggesting that Gata3 plays a role in this process (Zheng *et al.*, 2010). Although minimal Gata3 binding was detected in the promoter, CNS1 and CNS3 elements in the Foxp3 locus, the presence of functional Gata binding sites in the promoter and CNS1 may imply that Gata3 may be involved in regulating Foxp3 in other contexts as well.

Thus, the increased Gata3 noted in Bcl6-deficient Tregs can explain their increased expression of Foxp3 and Treg suppressor genes. The functional role of Gata3 in Foxp3 induction can explain increased Gata3 expression in wild-type Treg cells relative to conventional T cells as well as high Gata3 expression in Tregs at mucosal sites (Josefowicz *et al.*, 2012b). However, considering that Gata3 is the Th2 master lineage-specifying factor, it is important to understand what signals limit the transactivation of Th2 cytokines by Gata3 in Tregs, unlike conventional T cells. Gata3 induction in Tregs is guided by IL-2 and TCR signals, and is independent of IL-4/STAT6 signaling (Wohlfert *et al.*, 2011). Thus, our data with increased Th2 gene expression in Tregs in the absence of Bcl6 and the ability of Bcl6 to block transcriptional function of Gata3 indicates that Bcl6 is



essential to limit Gata3 expression in Tregs and their conversion to Th2-biased Tregs, defective in control of Th2 responses.



**Figure 26: Gata3 activation of Foxp3 promoter, independent of the palindromic Gata sites.**

(A) Schematic of transcription factor binding sites in the Foxp3 promoter depicting the palindromic GATA sites and the Foxp3 promoter constructs generated (WT Promoter with the GATA sites intact, Mut Promoter with the GATA sites deleted).

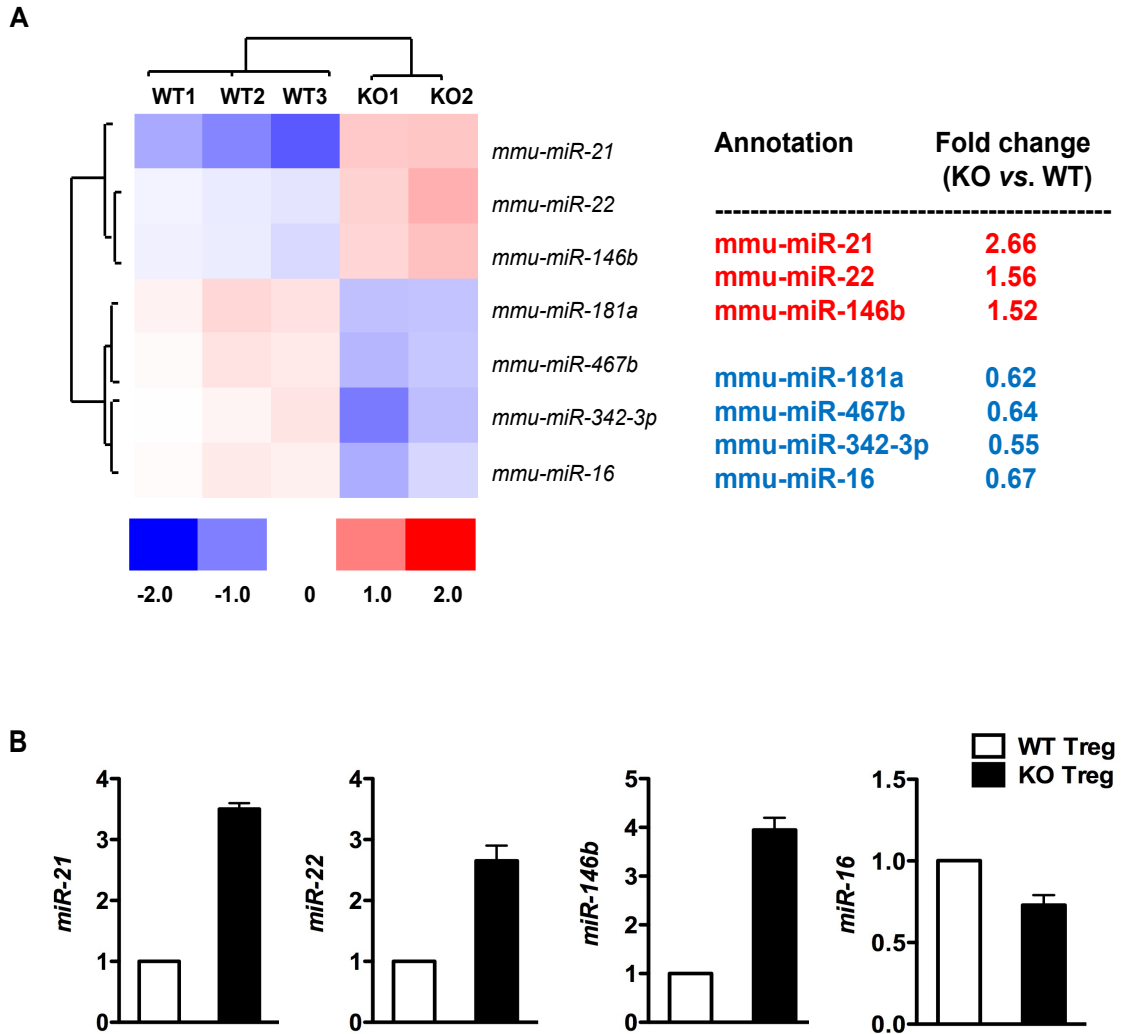
(B) Luciferase activity in Jurkat T cells co-transfected with the WT and Mut Foxp3 promoter driven luciferase reporters and expression constructs for CXN and CXN-Gata3. Following electroporation, cells were stimulated with PMA and Ionomycin 24 hrs prior to harvest and luciferase measurement. Results representative of 5 independent experiments.

## **Part II: Bcl6 repression of microRNA-21 is essential for stability of the Treg functional program**

### **Bcl6 represses expression of microRNAs in the Treg lineage**

MicroRNAs function as important regulators of T cell development and differentiation at the post-transcriptional level and are dysregulated in a number of autoimmune and inflammatory diseases (Cho, 2007; Jeker and Bluestone, 2010; O'Connell *et al.*, 2010). Treg cells exhibit a distinct Foxp3-dependent microRNA profile, with a number of miRNAs differentially expressed in Tregs compared to Tconv cells (Cobb *et al.*, 2006; Zhou *et al.*, 2008b). Mice deficient in the miRNA-processing enzymes, Dicer and Drosha in the Treg lineage, develop a severe multi-organ pathology, resembling Foxp3-deficient scurfy mice, highlighting the importance of miRNAs in Treg function (Liston *et al.*, 2008; Zhou *et al.*, 2008b). Dicer/Drosha-deficient Tregs exhibit developmental and functional defects and lack lineage stability, thus indicating that different miRNAs may regulate different aspects of Treg cell biology (Zhou *et al.*, 2008b). Indeed, miR-155 is important for Treg cell homeostasis (Lu *et al.*, 2009a), miR146a is critical for Treg control of Th1 responses (Lu *et al.*, 2010), miR-142-3p regulates cAMP production in Tregs (Huang *et al.*, 2009), and miR-10a marks stable Tregs (Jeker *et al.*, 2012). Thus, identifying the transcriptional control of these differentially expressed miRNAs in Tregs and their relevant target genes will help delineate the functional relevance of miRs in Tregs. MiR-146a targets STAT1 in Tregs; thus miR-146a-deficient Tregs exhibit a Th1-bias with increased STAT1 phosphorylation, secrete the Th1 cytokine IFN $\gamma$  and fail to suppress Th1

inflammatory responses (Lu *et al.*, 2010). Analogous to this situation, we have noted that in the absence of Bcl6, Tregs exhibit a profound Th2-bias, secrete Th2 cytokines and fail to control Th2 responses. We thus wondered if Bcl6 regulated Treg lineage stability by repressing micro-RNAs in Tregs. Importantly, studies on Bcl6 in Tfh cells have highlighted repression of miRNAs as an important aspect of Bcl6-mediated gene regulation in T cells (Yu *et al.*, 2009). Therefore, to identify miRNAs regulated by Bcl6 in Treg cells, we utilized a micro-RNA microarray approach, using RNA prepared from highly purified CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs from wild-type and Bcl6-deficient mice, activated *in vitro*. As shown in Figures 27A and 27B, three micro-RNAs were increased significantly in Bcl6-deficient Tregs, although only microRNA-21 (miR21) was increased more than 2-fold in the Bcl6-deficient Treg microRNA microarray. MiR-21 has previously been reported as increased in Treg cells compared to Tconv cells (Cobb *et al.*, 2006) and also linked to Foxp3 up-regulation in human Tregs, via unknown mechanisms (Rouas *et al.*, 2009). MiR-21 is also one of the most extensively studied micro-RNAs in the cancer field (Jung and Calin, 2010). In addition, it has been shown to promote heart disease (Thum *et al.*, 2008), which could be relevant to the myocarditis observed in Bcl6-deficient mice. Interestingly and highly relevant to our findings was its increased expression in Th2-mediated allergic airway inflammation (Lu *et al.*, 2009b). Two other micro-RNAs (miR-22 and miR-146b) were also increased in Bcl6-deficient Tregs relative to wild-type.



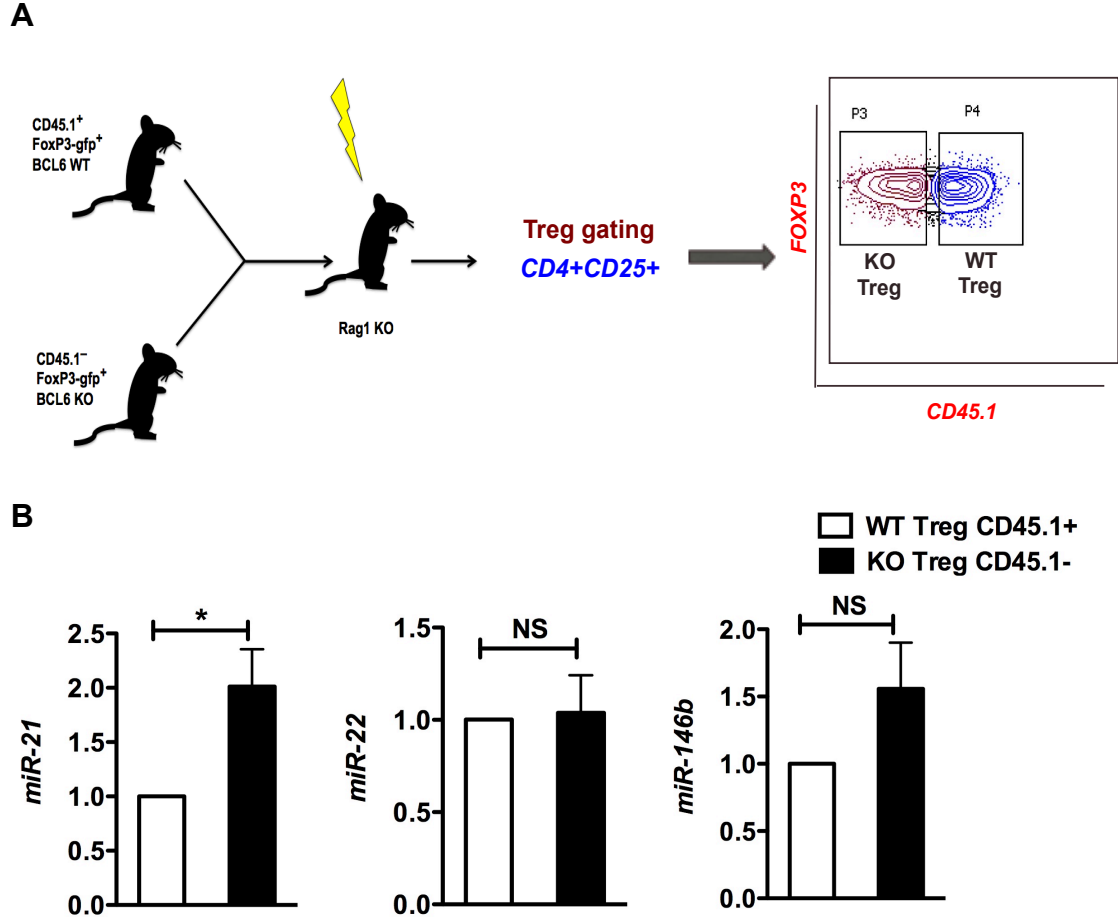
**Figure 27: Bcl6 represses miR-21 in Treg cells.**

(A) The heat map represents the miRNAs differentially expressed between sorted Bcl6-deficient (KO) and wild-type (WT) Tregs with statistical significance analyzed by expression microarrays. The color scale shown at the bottom illustrates the relative expression level of a miR across all samples: red color represents an expression level above mean, blue color represents expression level lower than the mean. n = Tregs from 3 wild-type mice and 2 Bcl6-deficient mice.

(B) Quantitative PCR validation of the selected set of miRNAs in Bcl6-deficient (KO) Tregs (black bars) relative to wild-type (WT) Tregs (white bars) following activation for 16 hrs with anti-CD3 and anti-CD28, normalized using sno202, sno234 and U6 as controls. Data are average expression from at least 3 mice per group.

### **Bcl6 intrinsically represses miR-21 in the Treg lineage**

In order to obtain a more definitive understanding of the regulation of miR-21 and Th2 genes in Bcl6-deficient Tregs by intrinsic and extrinsic mechanisms, we generated mixed bone marrow chimeras. Bone marrow from CD45.1<sup>+</sup> wild-type Foxp3-gfp mice was injected into irradiated Rag1-deficient recipient mice along with bone marrow from CD45.1<sup>-</sup> Bcl6-deficient Foxp3-gfp mice (Figure 28A). After 16-20 weeks, the chimeric mice were immunized with Ova-Alum to promote a Th2-type immune response, and CD45.1<sup>+</sup> wild-type Foxp3-gfp and CD45.1<sup>-</sup> Bcl6-deficient Foxp3-gfp Tregs were purified and tested for gene expression following activation *in vitro*. As shown in Figure 28B, miR-21 was significantly increased in Bcl6-deficient Tregs from the chimera compared to wild-type Tregs in the same chimera, confirming that miR-21 is an intrinsic target of Bcl6 in Tregs. MiR-22 and miR-146b were not increased in chimeric Bcl6-deficient Tregs and thus are not likely to be directly regulated by Bcl6 in Tregs. MiR-21 was specifically increased in Bcl6-deficient Tregs and not Tconv cells, suggesting that Bcl6 specifically represses miR-21 in the Treg lineage.



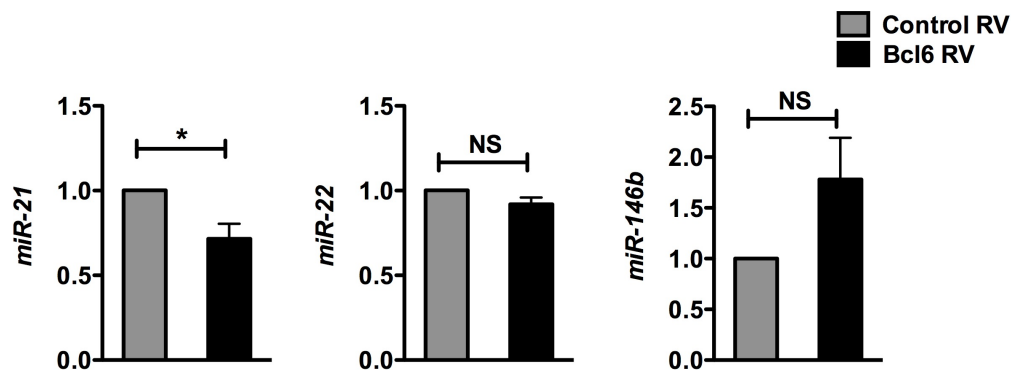
**Figure 28: Intrinsic regulation of miR-21 by Bcl6 in the Treg lineage.**

**(A)** Representative FACS sorting scheme for isolating Bcl6-deficient (KO) (CD45.1<sup>-</sup>) and wild-type (WT) (CD45.1<sup>+</sup>) Tregs from mixed bone marrow chimeras; with Tregs gated as CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells, prior to gating based on CD45.1 expression.

**(B)** Quantitative PCR (QPCR) analysis of miR transcripts in sorted bone marrow chimera-derived Bcl6-deficient (KO) Tregs (CD45.1<sup>-</sup>) and their respective wild-type Treg counterparts (CD45.1<sup>+</sup>), following activation for 16 hrs with anti-CD3 and anti-CD28. MiR21, miR22 and miR146b expression normalized to U6. Data are average expression from 6 different mice per group. \*p<0.05 (two-tailed Student's t-test) (error bars, s.e.m.)

### MiR-21 is a novel Bcl6 target gene in T cells

To further test the regulation of miR-21 by Bcl6 in T cells, we infected activated wild-type CD4<sup>+</sup> T cells, which have low levels of Bcl6, with a Bcl6-expressing retrovirus and tested repression of the miRNAs up-regulated in Bcl6-deficient Tregs (miR-21, miR-22 and miR-146b). As shown in Figure 29, Bcl6 specifically repressed miR-21 but not miR-22 or miR-146b, supporting the idea that miR-21, is a direct target of Bcl6 in T cells. This result is consistent with the miRNA regulation by Bcl6 reported by the Yu *et al* study (Yu *et al.*, 2009), which also noted miR-21 as one of the most strongly down-regulated miRNA by Bcl6 in T cells (~3.9 fold). Minimal effects were noted for the other two miRs (miR-146a was down-regulated ~1.1 fold while miR-22 was up-regulated ~1.2 fold). Thus, this result confirms miR-21 as a novel Bcl6 target gene in T cells, thereby warranting a functional assessment of miR-21 in Bcl6-mediated gene regulation in Tregs and T cells in general.



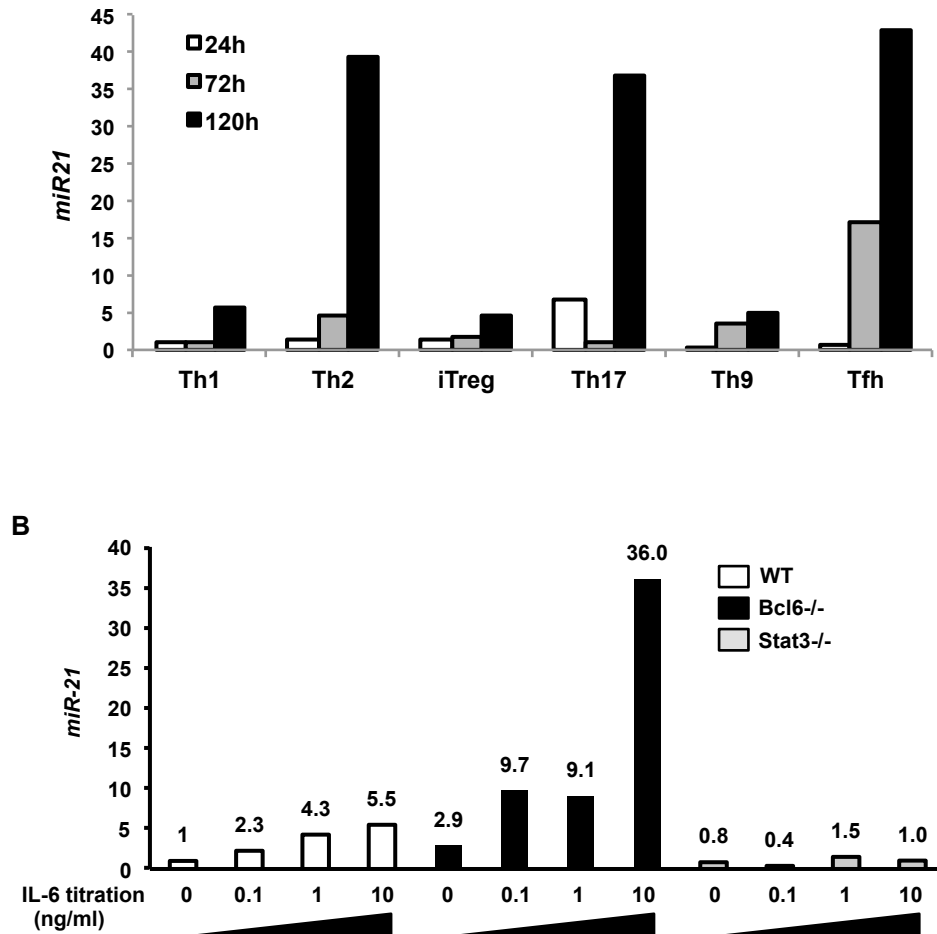
**Figure 29: MiR-21 is a novel Bcl6 gene target in T cells.**

Quantitative PCR analysis of expression of miRs – 21, 22 and 146b following ectopic expression of Bcl6 in T cells, normalized using sn0202, sno234 and U6 as controls. Naïve T cells were transduced with control RV and Bcl6 RV and the sorted RV<sup>+</sup> T cells were re-stimulated for 4-6 hrs to assess its effect on miR expression. Data are average expression from at least 3 different experiments. \*p<0.05, \*\*p<0.01 (two-tailed Student's t-test) (error bars=s.e.m.)

### **MiR-21 is up-regulated in Stat3-activating conditions**

The miR-21 gene promoter has binding sites for several transcriptional regulators - AP-1, Ets/PU.1, C/EBP $\alpha$ , NF $\kappa$ B, SRF, p53 and STAT3 (Fujita *et al.*, 2008). These enhancer elements are also conserved in the human miR-21 gene, indicating common transcriptional regulatory mechanisms for miR-21. AP-1 and Stat3 positively regulate miR-21 transcription via direct binding to the 3 AP-1 and 2 STAT3 binding sites in the miR-21 promoter (Fujita *et al.*, 2008; Loffler *et al.*, 2007). MiR-21 induction via Stat3 contributes to the oncogenic role of miR-21 in multiple myeloma and also links the role of miR-21 in cancer and inflammation (Iliopoulos *et al.*, 2010). Indeed, IL-21 acting via Stat3 up-regulates miR-21 in CD4 T cells as well as Sezary cells (T cell lymphoma) (van der Fits *et al.*, 2011). Consistent with a positive role for Stat3, we noted increased miR-21 in Th2 cells, as well as in Th17 and Tfh cells (Figure 30A), all cell types that are dependent upon Stat3 for their complete differentiation (Crotty, 2011; Ivanov *et al.*, 2007; Stritesky *et al.*, 2011). Treatment of wild-type, Stat3-deficient and Bcl6-deficient naïve CD4<sup>+</sup> T cells with increasing dose of IL-6 demonstrated the defective miR-21 induction of Stat3-deficient T cells. IL-6 induced miR-21 up to 5.5-fold in wild-type CD4 T cells, whereas in Bcl6-deficient CD4 T cells, basal miR-21 was increased about 3-fold and IL-6 titration further augmented it to almost 30-fold (Figure 30B). Thus, overall these results identify miR-21 as a Stat3 regulated factor in CD4 T cells and suggest potential counter-regulation of miR-21 transcription via Stat3 and Bcl6.





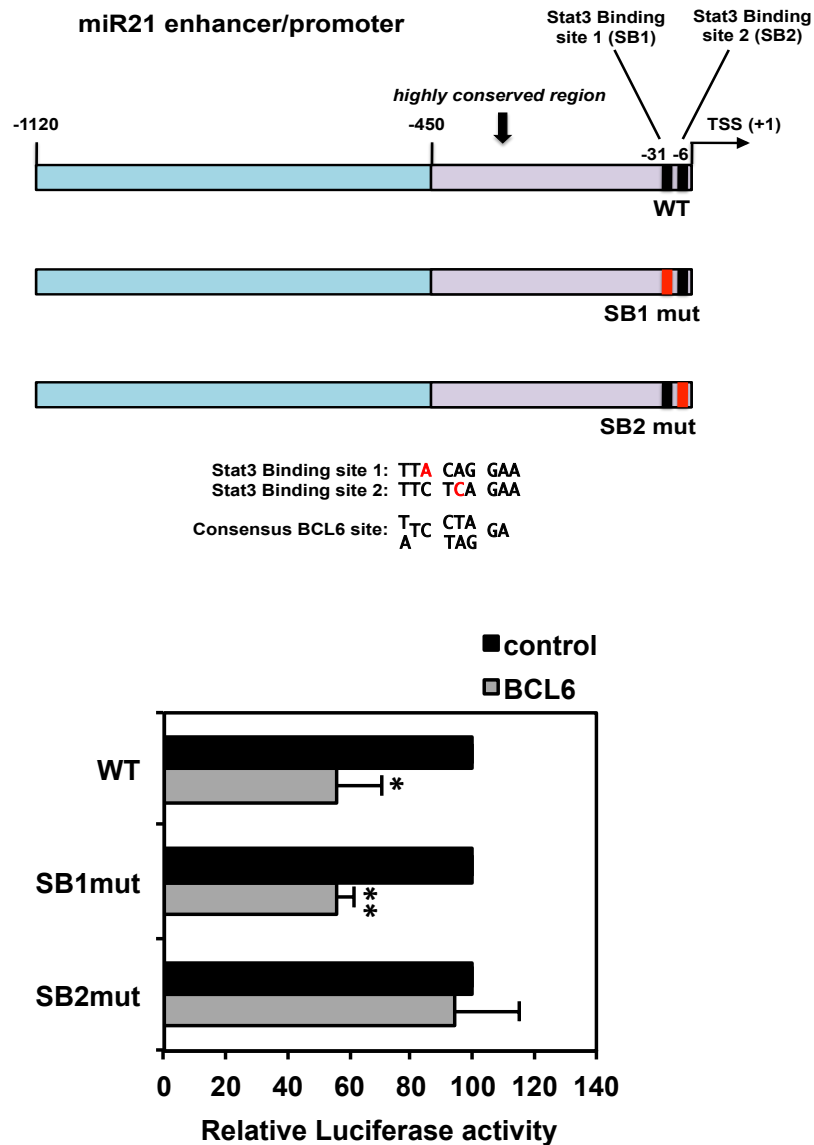
**Figure 30: MiR-21 is up-regulated in Stat3-activating Thelper conditions.**

**(A)** Quantitative PCR analysis of miR-21 expression during different time-points (24h, 72h and 120h) following culture of wild-type naïve T cells ( $CD4^+CD62L^+$ ) into different T helper subset differentiation conditions (Th1, Th2, iTreg, Th17, Th9 and Tfh), with expression normalized to U6. Data representative of 2 different mice.

**(B)** Quantitative PCR analysis of miR-21 expression following culture of WT, Bcl6-deficient and Stat3-deficient naïve T cells ( $CD4^+CD62L^+$ ) in response to increasing doses of IL-6 (0, 0.1, 1, 10 ng/ml) for 16-24 hrs, with expression normalized to U6. Data representative of 2 independent experiments.

### **Antagonistic regulation of miR-21 transcription by Bcl6 and Stat3**

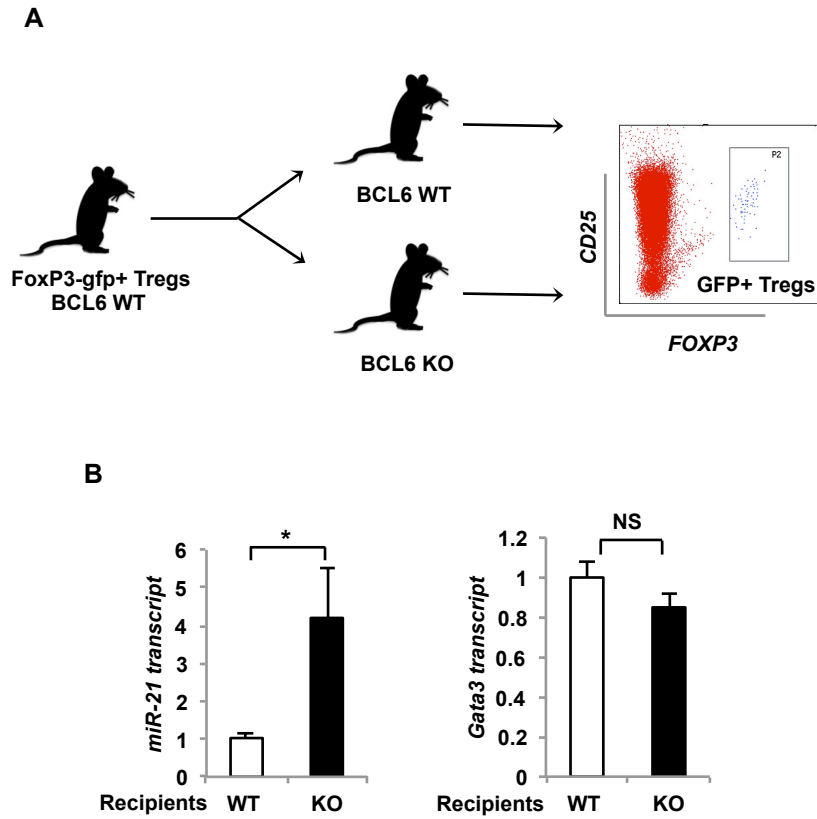
The Bcl6 recognition motif resembles binding sites for the cytokine-induced STAT family of transcription factors and Bcl6 has been shown to repress STAT-induced cytokine transcription (Shaffer *et al.*, 2000). Since our results identify miR-21 as a target gene for Bcl6, we next wanted to assess if Bcl6 counter-regulated miR-21 via competing with Stat3 for the two well characterized Stat3-binding sites in the miR-21 promoter. To test this regulation, we generated three different miR-21 promoter constructs – full-length (WT) construct with the two Stat3-binding sites intact, SB1 mutant with the 5' Stat-3 binding site mutated and an SB2 mutant with the 3' Stat3-binding site mutated by site-directed mutagenesis (Figure 31). These constructs were tested in a transient transfection assay in Jurkat T cells, with or without Bcl6 expression plasmids. We found that Bcl6 potently repressed the wild-type miR-21 promoter. Mutation of the 5' Stat3-binding site (SB1) had no effect on repression by Bcl6, whereas mutation of the 3' Stat3-binding site (SB2), completely abolished repression by Bcl6 (Figure 31). By comparing the sequence of the two Stat3 binding sites, we found that the SB2 site has better consensus to the core Bcl6 recognition motif than SB1, thus contributing to the differential effect of Bcl6 in regulating miR-21 via the two sites. These data suggest that Bcl6 and Stat3 may compete for binding to the miR-21 promoter, and that each opposes the function of the other factor. Thus, Bcl6 and Stat3 have opposing effects on the transcription of miR-21 in T cells.



**Figure 31: Bcl6 directly targets miR-21 via binding sites in its promoter.** Luciferase activity in Jurkat T cells co-transfected with full-length or SB1 and SB2 mutated miR21 promoter driven luciferase reporters and expression constructs for CXN and CXN-Bcl6. Cells were stimulated with PMA and Ionomycin for 24 hrs prior to harvest and luciferase measurement. Results averaged from 5 independent experiments, where the basal activity of each promoter construct is set to 100 relative units. \* $p < 0.05$ , \*\* $p < 0.01$  (two-tailed Student's t-test) (error bars=s.e.m.)

### **Inflammatory signals contribute to miR-21 up-regulation in Tregs**

Since Stat3 induces miR-21 during inflammation (Iliopoulos *et al.*, 2010), and miR-21 induction is associated with Th2 inflammation (Lu *et al.*, 2009b), we next wondered if some of the increase in miR-21 noted in Bcl6-deficient Tregs was due to the Th2 inflammatory disease that occurs in Bcl6-deficient mice. Therefore, to address the potential extrinsic regulation of miR-21 by the inflammatory cues in Bcl6-deficient mice, we transferred wild-type Foxp3-gfp Tregs into wild-type or Bcl6-deficient mice, immunized with HEL/Alum to induce a Th2 response, then after 3 days, isolated the Tregs by sorting for GFP expression (Figure 32A). As shown in Figure 32B, in comparison to transfers into wild-type mice, wild-type Tregs that were transferred into Bcl6-deficient mice showed a significant increase in miR-21. However, Gata3 mRNA was not increased in the wild-type Tregs in the Bcl6-deficient background, and Th2 cytokines were not detected in the wild-type Tregs in either the wild-type or Bcl6-deficient background (Figure 32B, data not shown). These data indicate that in addition to being a Bcl6 target, miR-21 expression in Tregs is also regulated extrinsically by inflammation. At the same time, failure of wild-type Tregs to up-regulate Th2 genes in the Bcl6-deficient environment highlights the Treg-intrinsic role of Bcl6 in controlling Gata3 and Th2 cytokines.



**Figure 32: Extrinsic regulation of miR-21 in Tregs by inflammatory cues in Bcl6-deficient mice.**

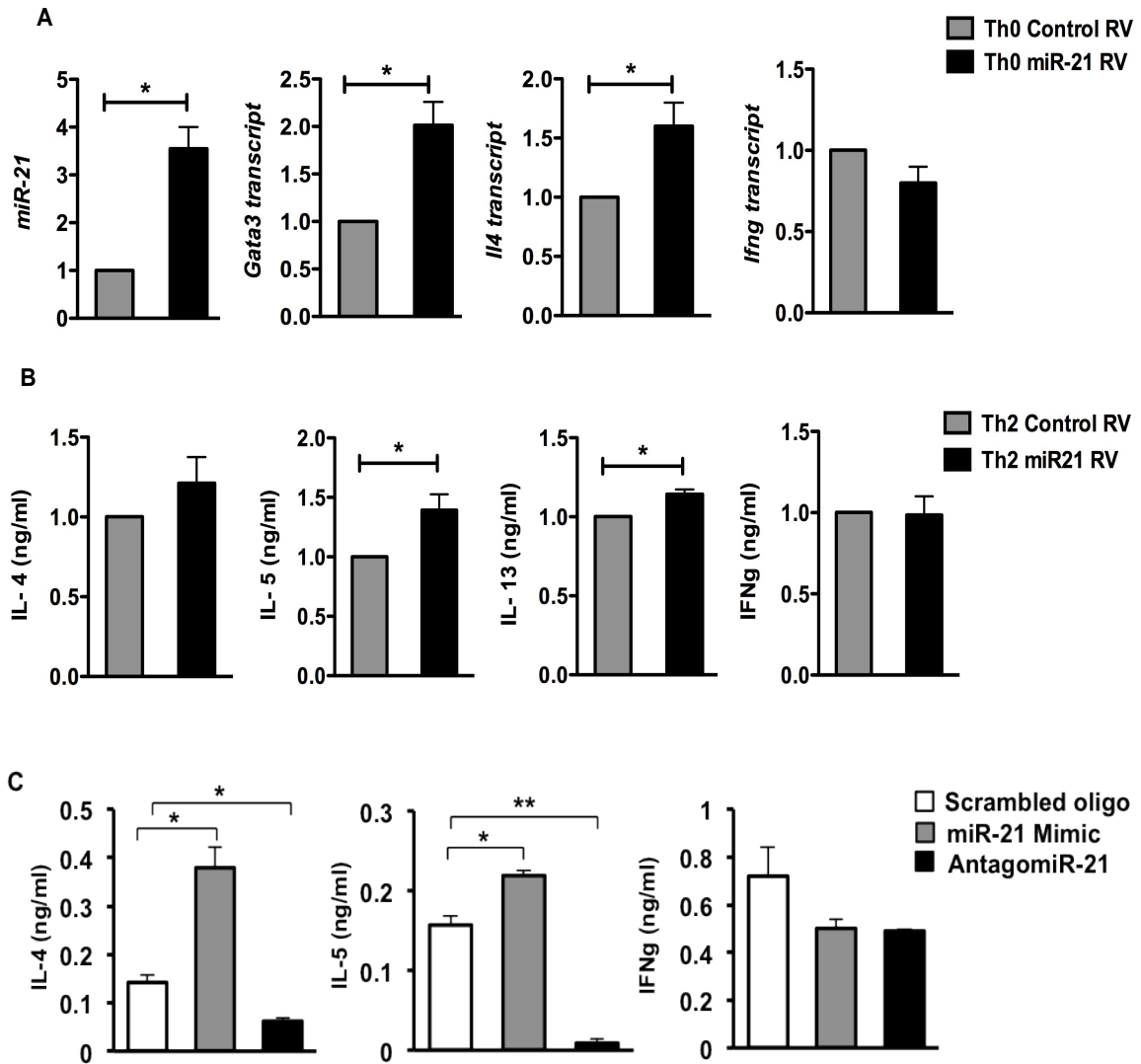
**(A)** Representative FACS sorting scheme for isolating WT CD25<sup>+</sup>Foxp3<sup>+</sup> (GFP<sup>+</sup>) Tregs 3 days following adoptive transfer i.p. into Bcl6-deficient (KO) (black bars) and wild-type (white bars) recipients and immunized next day.

**(B)** Quantitative PCR analysis of miR-21 and *Gata3* transcript in FACS-sorted wild-type CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs isolated from WT and Bcl6-deficient mice, following activation for 16 hrs with anti-CD3 and anti-CD28 with miR expression normalized to U6, and *Gata3* expression normalized to *tubb5*. Data are average of 3-4 mice/group.

### **MiR-21 can promote Th2 differentiation in a T cell-autonomous manner**

Although miR-21 has been linked to Th2 inflammation, it was reported as primarily elevated in cells of the myeloid lineage (Lu *et al.*, 2011b; Lu *et al.*, 2009b). MiR-21 was demonstrated to promote Th2 differentiation indirectly via inhibition of the myeloid cell derived Th1 polarizing cytokine, IL-12. Thus, whether miR-21 regulates Th2 differentiation in a T cell intrinsic manner has not been ascertained. To test this idea, we constructed a miR-21-expressing retrovirus, and used it to infect “naïve” CD4<sup>+</sup>CD62L<sup>+</sup> T cells from wild-type mice, with the hypothesis that over-expression of miR-21 in T cells by retrovirus could recapitulate the elevated levels of miR-21 observed in Bcl6-deficient Tregs. As shown in Figure 33A, the retrovirus promoted a greater than 3-fold increase in miR-21 when expressed in T cells, which was comparable to the increased miR-21 level observed in Bcl6-deficient Tregs. We then tested whether miR-21 could induce Th2 differentiation in cells cultured under “Th0” conditions, where no cytokines were added. As shown in Figure 33A, miR-21 promoted increases in Th2 genes (*Gata3*, *Il4* and *Il13*), whereas expression of the Th1 cytokine *Ifng* was mildly repressed by miR-21. We also found that miR-21 could augment Th2 but not Th1 cytokine expression when over-expressed in Th2 cells (Figure 33B). To further assess miR-21 activity in promoting Th2 differentiation, we tested a synthetic miR-21 “mimic” as well as an anti-sense miR-21 inhibitor “antagomiR-21” in Th0 differentiation cultures. As shown in Figure 33C, the mimic significantly promoted Th2 differentiation whereas the antagomiR-21 inhibited Th2 differentiation, as measured by IL-4 and IL-5 secretion. The mimic and

inhibitor did not significantly affect IFN $\gamma$ , although there was a trend towards decreased IFN $\gamma$  with both treatments. These data show for the first time that miR-21 can promote Th2 differentiation by a T cell intrinsic mechanism. Thus, increased miR-21 in Bcl6-deficient Tregs may thus be a causal factor for the abnormal expression of Th2 genes in these cells. Reduced Th2 differentiation and allergic airway inflammation and heightened Th1 responses were reported for miR-21-deficient mice (Lu *et al.*, 2011b). Our results with a positive role for miR-21 in promoting Th2 differentiation are consistent with the observations by Lu *et al.* Importantly, our results identify a novel T cell autonomous regulation of Th1 vs. Th2 bias by miR-21, that fits with the post-transcriptional control of Th2 differentiation by Bcl6 and can also mechanistically explain the observed Th2-bias noted for Bcl6-deficient Treg cells.



**Figure 33: MiR-21 promotes Th2 differentiation of naïve T cells in a T cell-intrinsic manner.**

**(A)** Quantitative PCR analysis of miR-21 and *Gata3*, *Il4*, *Il13* and *Ifng* following ectopic expression of miR-21 RV in non-polarized (Th0) T cells, relative to control RV transduced cells. Sorted RV<sup>+</sup> cells were re-stimulated with anti-CD3 and anti-CD28 for 4-6 hrs for gene expression analysis. MiR expression normalized to U6, and expression of other genes normalized to  $\beta$ -tub. Data are averaged from at least 3 independent experiments.

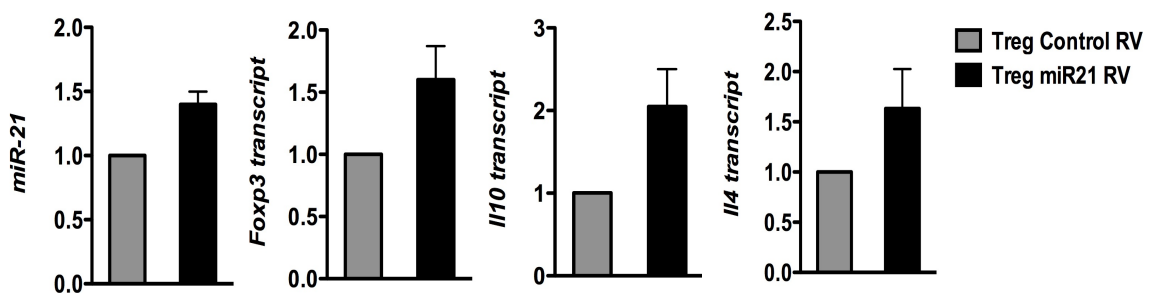
**(B)** ELISA for cytokines assayed from supernatants of miR-21 transduced Th2 cells (IL-4 – 20 ng/ml plus anti-IFN $\gamma$  - 10  $\mu$ g/ml), relative to control RV transduced cells. Cells were re-stimulated with anti-CD3 and anti-CD28 for 24 hrs following sorting of RV<sup>+</sup> Th2 cells for cytokine measurements. \*p<0.05, (two-tailed Student's t-test) (error bars=s.e.m.)

**(C)** ELISA for cytokines assayed from supernatants of scrambled control, miR-21 mimic and antagomiR-21 treated naïve T cells. Cells were cultured with the oligos (1  $\mu$ M) over a 5-day period, then re-stimulated with anti-CD3 and anti-CD28 overnight for cytokine measurements. Data are representative of 3 independent experiments.



## MiR-21 up-regulates Treg and Th2 genes in the Treg lineage

Since ectopic expression of miR-21 augments Th2 gene expression in T cells, we next wanted to address if miR-21 over-expression can induce a similar Th2 bias in Treg cells. MiR-21 is one of the miRNAs up-regulated in Tregs compared to Tconv cells (Cobb *et al.*, 2006) and miR-21 is reported to positively regulate *Foxp3* expression in human Tregs, although the mechanism is yet unknown (Rouas *et al.*, 2009). We overexpressed miR-21 using a retroviral approach in wild-type Tregs and noted a similar up-regulation of Treg genes (*Foxp3* and *Il10*) and Th2 cytokine (*Il4*) expression with miR-21 RV compared to control RV (Figure 34). Interestingly, when these miR-21 RV transduced wild-type Tregs were tested in a standard Treg suppression assay with wild-type CD25<sup>-</sup> Tresp cells and APCs, the miR-21 RV Tregs exhibited a trend towards increased suppressor function compared to control RV Tregs (data not shown). Thus, ectopically expressed miR-21 up-regulates both Treg and Th2 genes in the Treg lineage. This indicates that the increased miR-21 may serve as a causal factor for the increased Th2 genes, and also contribute to the up-regulated Treg genes and enhanced suppressor function *in vitro*, noted for the Bcl6-deficient Treg cells.

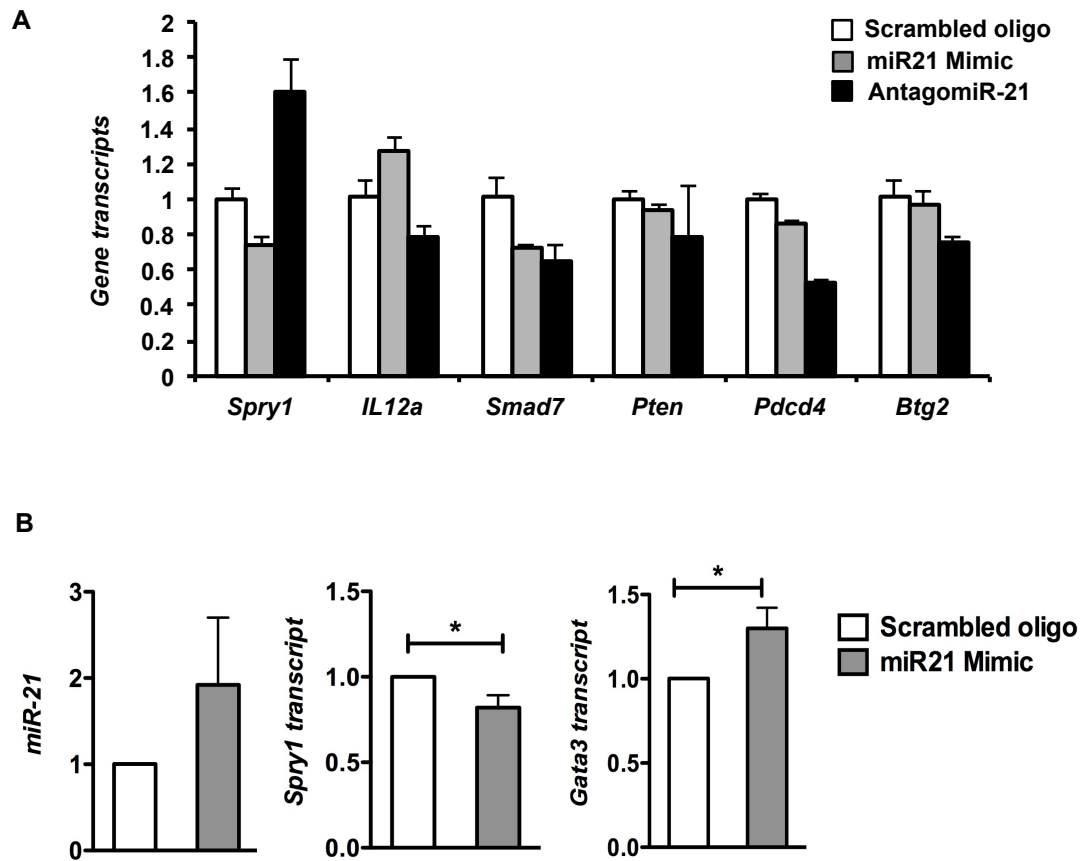


**Figure 34: MiR-21 augments Treg and Th2 genes in Treg cells.**

Quantitative PCR analysis of miR-21, *Foxp3*, *Il10* and *Il4* in miR-21 RV and control RV transduced wild-type (WT) Tregs, following 4-6 hrs activation with anti-CD3 and anti-CD28. MiR-21 expression normalized to U6 and other genes normalized to *tubb5* as control.

## **MiR-21 target genes in T cells that relate to its role in promoting Th2 immune responses**

The advantage of using the synthetic oligos to overexpress or inhibit miR-21 is that we could better control the timing of miR21 expression, and thus we treated naïve wild-type CD4 T cells with miR-21 mimic, antagomiR-21 or scrambled control, and assessed downstream miR-21 targets after 12-16 hrs of expression. A large number of miR-21 target genes have been described (Jung and Calin, 2010), and we analyzed six of the most well-known target mRNAs (Figure 35A). Of these six genes, only Sprouty1 (*Spry1*), a negative regulator of the MAP kinase pathway (Thum *et al.*, 2008), was consistently decreased by mimic treatment (Figure 35B). At the same time, the miR-21 mimic augmented Gata3 mRNA (Figure 35B). Since the MAP kinase pathway has been linked to promoting Gata3 protein stability (Yamashita *et al.*, 2005), and that Gata3 can auto-activate its own expression (Ouyang *et al.*, 2000), these data indicate that miR-21 might augment Gata3 expression by decreasing *Spry1* and increasing MAP kinase activity.



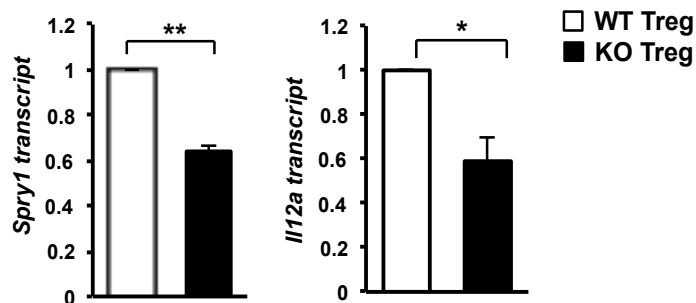
**Figure 35: MiR-21 gene target in T cells (*Spry1*) linked to its role in promoting Th2 differentiation.**

**(A)** Quantitative PCR analysis of miR-21 gene targets (*Spry1*, *IL12a*, *Smad7*, *Pten*, *Pdc4*, *Btg2*) following 12-16 hrs treatment of naïve T cells with scrambled control, miR-21 mimic and antagomiR-21 oligos (1  $\mu$ M), normalized to *tubb5* as control.

**(B)** Quantitative PCR analysis of miR-21, *Spry1* and *Gata3* following 12-16 hrs treatment of naïve T cells with scrambled control and miR-21 mimic (1  $\mu$ M). MiR expression normalized to U6, and expression of other genes normalized to *tubb5*. Data are averaged from at least 3 independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$  (two-tailed Student's t-test) (error bars, s.e.m.)

## MiR-21 target genes in Treg cells

To validate these miR-21 targets in Treg cells, we next tested miR-21 target genes in wild-type versus Bcl6-deficient Tregs (Figure 36). We observed a decrease of *Spry1* mRNA in Bcl6-deficient Tregs compared to the wild-type Tregs. In addition, we observed a strong decrease in *Il12a* mRNA in Bcl6-deficient Tregs compared to the wild-type Tregs. *Il12a* is a component of the key Treg suppressive cytokine IL-35, in conjunction with Ebi3 (Collison *et al.*, 2007). Our results suggest that the decrease in *Il12a* mRNA in Bcl6-deficient Tregs may lead to less production of IL-35, which could contribute to the inability of Bcl6-deficient Tregs to suppress Th2 inflammation. IL-35 has been reported to control Th2 inflammatory responses in a house-dust mite allergen challenge model *in vivo* (Huang *et al.*, 2011). Thus, a critical target of miR-21 in Tregs appears to be an important immuno-suppressive cytokine.



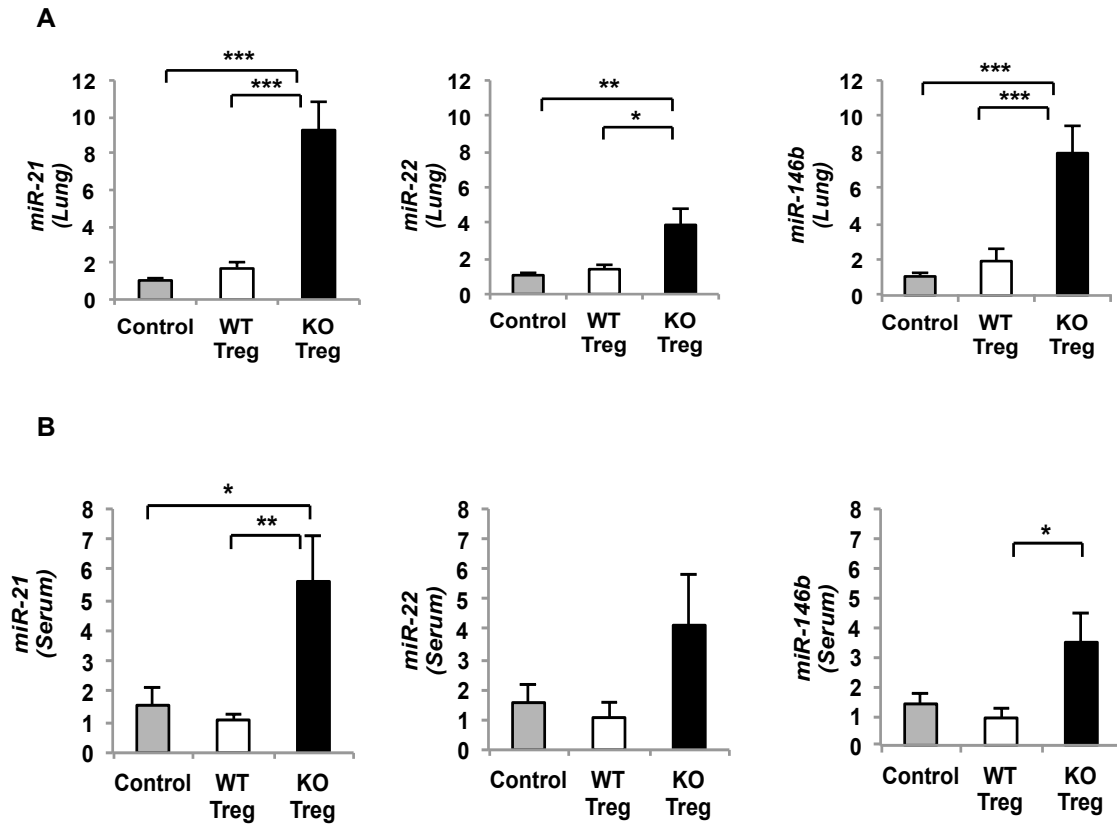
**Figure 36: *Spry1* and *Il12a* are miR-21 gene targets in Treg cells.**

Quantitative PCR analysis of *Spry1* and *Il12a* in sorted Tregs from Bcl6-deficient (KO) (black bars) and wild-type (WT) (white bars) Foxp3-gfp mice, with expression normalized to  $\beta$ -*tub*. n = 3 mice per group.

\*p<0.05, \*\*p<0.01(two-tailed Student's t-test) (error bars, s.e.m.)

### **Increased miR-21 associated with exacerbated Th2 inflammatory responses in lungs of mice in the airway inflammation model**

Since miR-21 is up-regulated in mouse models of allergen-driven Th2-type inflammation (Lu *et al.*, 2009b), we next tested if miR-21 was up-regulated in the lungs of the mice in which we induced Th2 airway inflammation. While miR-21 expression was not different between control and the wild-type Treg-treated mice, miR-21 was significantly increased in the total RNA of lungs taken from the Bcl6-deficient Treg-treated mice (Figure 37A). These data are consistent with previous results that Bcl6-deficient Tregs promote severe Th2 inflammation, rather than repress inflammation. While miR-146b was also seen up-regulated in Th2 inflammation, changes in miR-22 were not observed (Garbacki *et al.*, 2011; Lu *et al.*, 2009b). Strikingly, miR-22 and miR-146b followed a similar pattern of expression in the lungs as miR-21 (Figure 37A), indicating expression of miR-21, miR-22 and miR146b might be a unique micro-RNA signature induced by Bcl6-deficient Tregs in Th2-type inflammation. We wondered if the increase in miR-21 in the lungs that received Bcl6-deficient Tregs correlated with circulating levels of miR-21, as has been seen in cancer (Asaga *et al.*, 2011; Tomimaru *et al.*, 2012; Wei *et al.*, 2011). Indeed, in mice that received Bcl6-deficient Tregs, we detected significantly elevated levels of miR-21 in serum (Figure 37B). We also observed elevated serum levels of miR-22 and miR-146b, though to a lesser degree than for miR-21. Nonetheless, these results indicate that increased serum miR-21 may represent a novel biomarker for Th2-type inflammatory diseases.

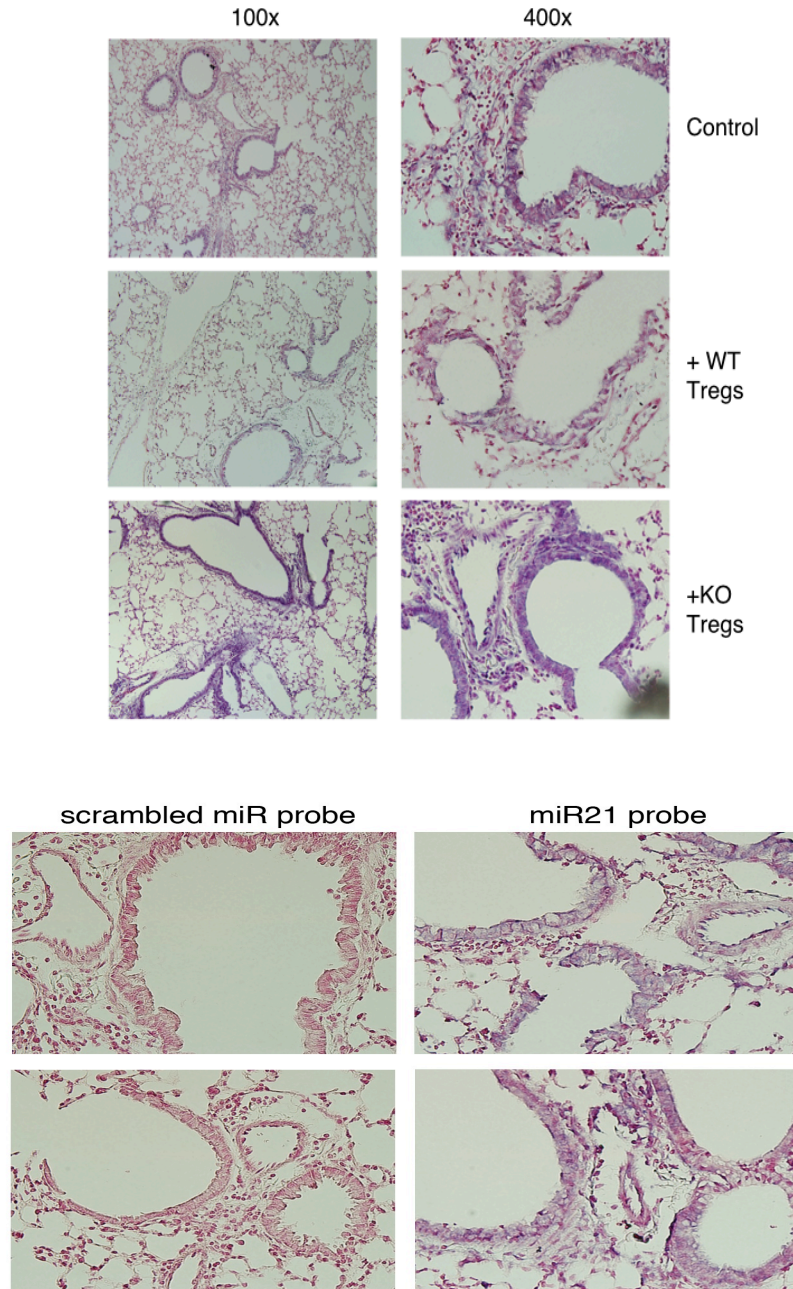


**Figure 37: Increased miR-21 in lungs and sera of mice receiving Bcl6-deficient Tregs in the airway inflammation model.**

Quantitative PCR analysis of the 3 miRs up-regulated in Bcl6-deficient (KO) Tregs in the total lung RNA (**A**) and sera (**B**) of Ova-sensitized and intranasally challenged wild-type (WT) recipient mice (grey bars) or immunized i.p. with Bcl6-deficient Tregs (black bars) or wild-type Tregs (white bars) normalized using U6 as control.

### **Cell-types expressing miR-21 in allergic airway inflammation model**

We next tested whether cell types other than Tregs expressed the high levels of miR-21 seen in mice that received Bcl6-deficient Tregs. A previous study analyzing allergic airway inflammation showed that miR-21 was primarily expressed in myeloid cells in the inflamed lung (Lu *et al.*, 2009b). As shown in Figure 38, we used *in situ* hybridization to analyze miR-21 expression in the lungs of mice in our allergic airway inflammation model. While we observed miR-21 expression in myeloid cells in the inflammatory lesions of the lungs, we observed the strongest expression of miR-21 in airway epithelium. The elevated expression of miR-21 in the total lungs of mice receiving Bcl6-deficient Tregs therefore correlated with more myeloid cells as well as higher expression of miR-21 in airway epithelium. These results indicate that an interplay between the Bcl6-deficient Tregs and the airway epithelium leads to greater microRNA expression, which further correlates with increased amounts of circulating miR-21. The increased circulating microRNA may be due to exosomes shed from miR-expressing cells (Hunter *et al.*, 2008; Taylor and Gercel-Taylor, 2008).



**Figure 38: Increased miR-21 expression in the lung epithelial cells in mice receiving Bcl6-deficient Tregs.**

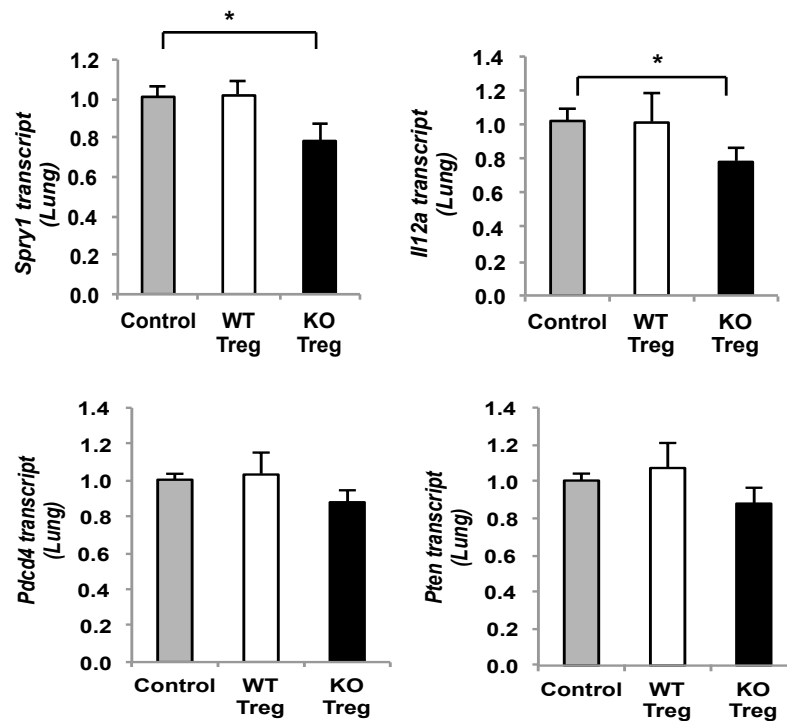
**(Top)** Expression of miR-21 in the paraffin-embedded lung sections of Ova-sensitized and intranasally challenged wild-type recipient mice or immunized i.p. with Bcl6-deficient Tregs or wild-type Tregs as determined by LNA-based *in situ* hybridization. (*Left*, 100X; *Right*, 400X). Sections are representative of hybridization results from at least 3 different experiments.

**(Bottom)** Expression of scrambled control probe and miR-21 probe in the paraffin-embedded lung sections of Ova-sensitized and intranasally challenged wild-type recipient mice immunized i.p. with Bcl6-deficient Tregs in 2 representative sections as determined by LNA-based *in situ* hybridization.



## MiR-21 target genes associated with exacerbated Th2 inflammatory responses in the airway inflammation model

Next, we wanted to validate the miR-21 gene targets in the airway inflammation model where the Bcl6-deficient Tregs promoted increased miR-21 and exacerbated Th2 responses. Strikingly, we found that both *Spry1* and *Il12a* mRNAs were significantly decreased in lungs of mice that received Bcl6-deficient Tregs, whereas other miR-21 target mRNAs were not significantly decreased (Figure 39). This confirms that *Spry1* and *Il12a* are relevant target genes that provide a potential mechanism for miR-21 in promoting Th2 inflammatory responses *in vivo*.



**Figure 39: *Spry1* and *Il12a* are relevant miR-21 gene targets in the airway inflammation model.**

Quantitative PCR validation of *Spry1*, *Il12a*, *Pdc4* and *Pten* in the total lung RNA of Ova-sensitized and intranasally challenged wild-type recipient mice (grey bars) or immunized i.p. with Bcl6-deficient (KO) Tregs (black bars) or wild-type (WT) Tregs (white bars) normalized using *tubb5* as control.

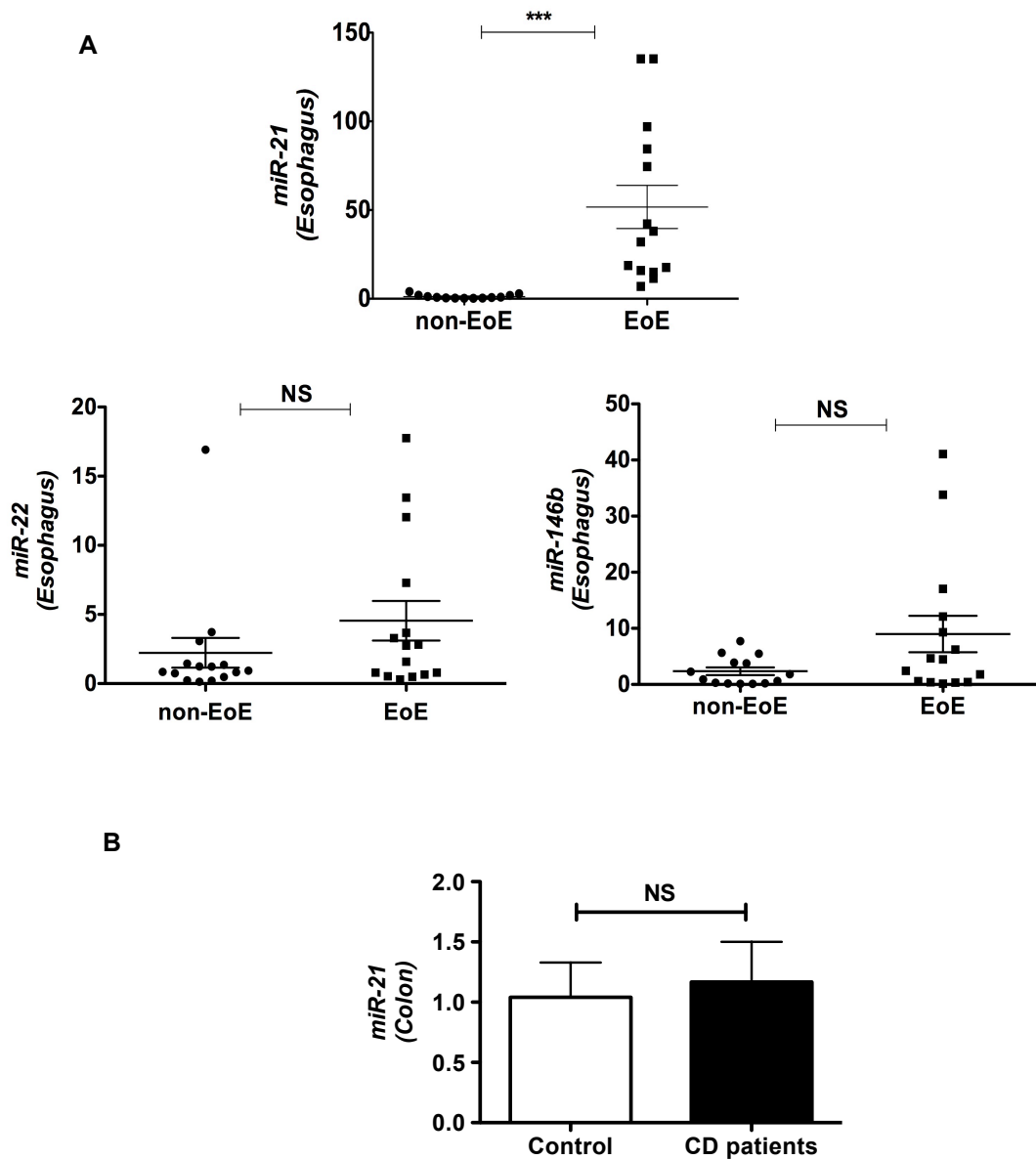
n = 8-10 mice per group. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 (two-tailed Student's t-test) (error bars, s.e.m.)

**Part III: MiR-21 as a clinically relevant non-invasive biomarker for human Th2 inflammatory conditions**

**MiR-21 is strongly elevated in biopsies of pediatric patients with Eosinophilic Esophagitis (EoE)**

MiR-21 is one of the most commonly dysregulated miRNA in a wide variety of cancers (Hatley *et al.*, 2010; Jung and Calin, 2010). It is also reported as increased in heart diseases (Thum *et al.*, 2008), lung fibrosis (Liu *et al.*, 2010), type 1- diabetes (Ruan *et al.*, 2011), T cell lymphoma (van der Fits *et al.*, 2011), lupus (Garchow *et al.*, 2011; Pan *et al.*, 2010), and psoriasis (Meisgen *et al.*, 2012; Zibert *et al.*, 2010). Relevant to the role of miR-21 in promoting Th2 responses, miR-21 is up-regulated in three different mouse models of allergic inflammation (OVA, IL-13 transgenic and *Aspergillus fumigatus*) (Lu *et al.*, 2009b). Further, we have also noted a strong induction of miR-21 in our airway inflammation studies, particularly for mice receiving Bcl6-deficient Tregs that developed severe Th2 inflammation. While inhibition of the Th1 cytokine IL-12 has been demonstrated as one mechanism for the association of miR-21 in Th2 diseases (Lu *et al.*, 2011b; Lu *et al.*, 2009b), our studies highlight a novel T cell intrinsic role for miR-21 in Th2 differentiation. Thus overall these studies suggest that miR-21 may not just be a passive marker up-regulated in Th2 diseases, but serves a functional role in augmenting Th2 responses via its role in myeloid cells and T cells. Thus, miR-21 may be part of a positive feedback loop amplifying the Th2 inflammatory responses.

MiRNAs are being used as diagnostic biomarkers for cancer and a number of autoimmune and inflammatory pathologies (O'Connell *et al.*, 2012; Sonkoly and Pivarcsi, 2009; Tricoli and Jacobson, 2007). Thus, to pursue the idea of miR-21 as a novel biomarker for Th2-type inflammatory diseases, we wondered if miR-21 was up-regulated in human allergic diseases. Eosinophilic esophagitis (EoE) is an increasingly prevalent allergic disease where the esophagus becomes highly inflamed, frequently in response to food allergens (Liacouras *et al.*, 2011; Noel and Rothenberg, 2005; Rothenberg, 2009). Further, EoE is a Th2-type inflammatory disease characterized by high eosinophil infiltration into the esophageal epithelium, with the hallmark Th2 cytokines implicated in the pathology. We therefore obtained esophageal biopsies from pediatric EoE patients (Subbarao *et al.*, 2011). Our controls in the study were patients who had undergone esophagogastroduodenoscopy (EGD) for a variety of non-specific reasons but had normal histology of biopsies from the upper gastro-intestinal tract. MiR-21 was elevated an average of 50-fold in EoE biopsies over control biopsy levels, with statistical significance. In contrast, miR-22 and miR-146b were not elevated in EoE biopsies (Figure 40A). To assess the specificity of miR-21 induction in Th2 diseases, we also tested biopsies from human patients with Crohn's disease, a Th1-mediated disease (Dalal and Kwon, 2010). MiR-21 levels were unchanged in the biopsies of Crohn's patients compared to controls, while miR-146b was undetected (Figure 40B). These results indicate that miR-21 is specifically elevated in human Th2 inflammatory diseases and could serve as a potential biomarker for Th2 conditions.

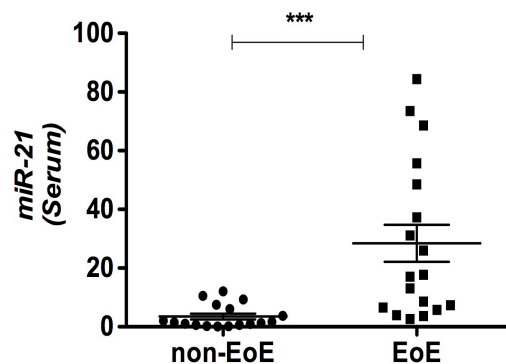


**Figure 40: Increased miR-21 in the biopsies of pediatric patients with Eosinophilic Esophagitis (EoE).**

(A) Quantitative PCR analysis of miR-21, miR-22 and miR-146b expression in esophageal biopsy RNA from healthy controls and eosinophilic esophagitis (EoE) patients, normalized using U6 as control. n = 18-20 subjects per group. (B) Quantitative PCR analysis of miR-21 expression in colon biopsy RNA from healthy controls and Crohn's disease (CD) patients, normalized using U6 as control. n=2 controls, 7 CD patients. \*\*\*p<0.001 (two-tailed Student's t-test) (error bars, s.e.m.)

## Circulating miR-21 can serve as a non-invasive biomarker for human Th2 inflammatory diseases

Circulating miRNAs are being used as non-invasive biomarkers in the cancer field (Cho, 2007). Since we noted increased circulating miR-21 in the sera of mice receiving Bcl6-deficient Tregs that correlated with a striking increase in miR-21 in the lungs and Th2 exacerbation, we tested sera of EoE patients. Indeed, consistent with the increase in miR-21 in the EoE biopsies, we noted an almost 30-fold increase in circulating miR-21 in the sera of EoE patients compared to controls (Figure 41). The other control miRs – 22 and 146b were not detected in the sera samples. This suggested that circulating miR-21 can be used as a non-invasive biomarker for Th2-type inflammation. Another report also noted increased miR-21 in the biopsies of EoE patients (Lu *et al.*, 2012a; Lu *et al.*, 2012b), although they noted only a 4-fold increase and reported little to no levels of circulating miR-21 in EoE (Lu *et al.*, 2012b).



**Figure 41: Increased miR-21 in the sera of pediatric patients with Eosinophilic Esophagitis (EoE).**

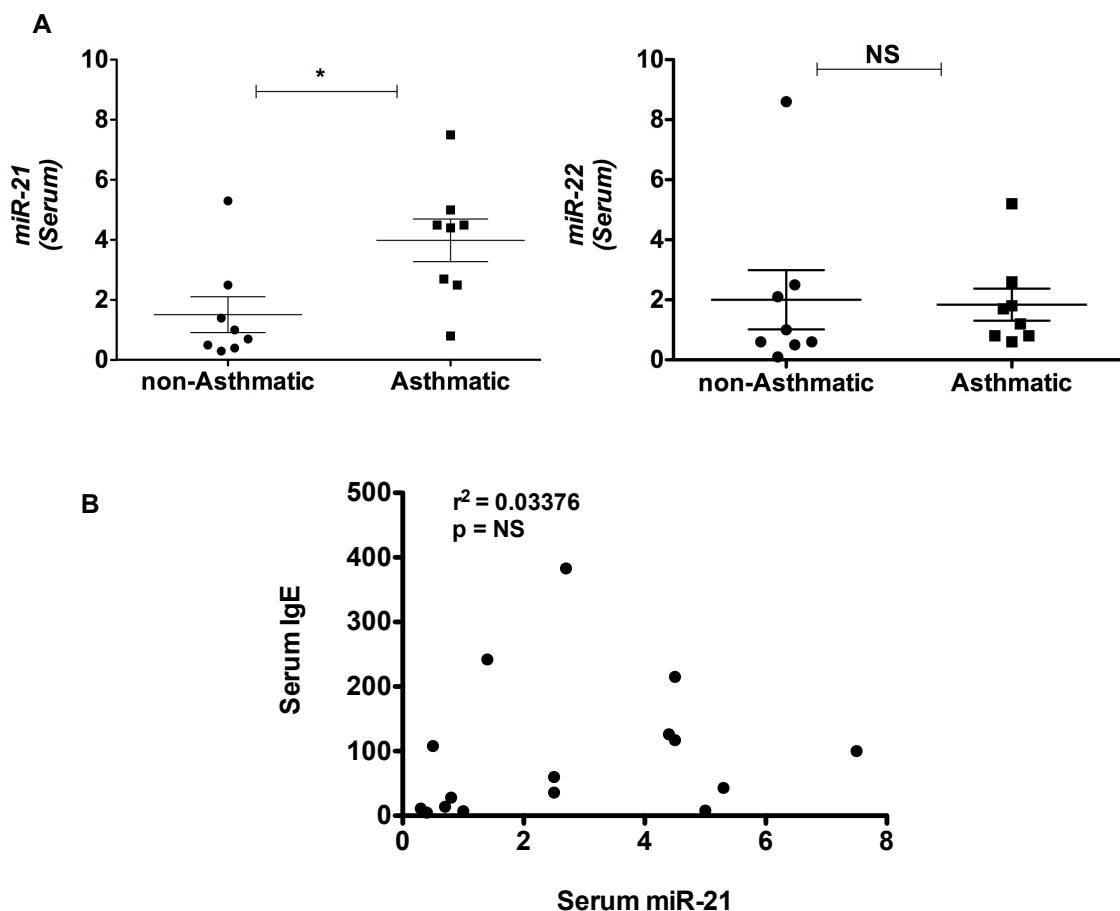
Quantitative PCR analysis of miR-21 expression in serum RNA from healthy controls and eosinophilic esophagitis (EoE) patients, normalized using U6 as control. n = 18-20 subjects per group.

\*p<0.05, \*\*p<0.01, \*\*\*p<0.001 (two-tailed Student's t-test) (error bars, s.e.m.)

### **Increased circulating miR-21 in the sera of asthmatic patients, however, miR-21 does not correlate with atopy**

Some explanations for the differences between our results and the other studies (Lu *et al.*, 2012a; Lu *et al.*, 2012b) assessing miR-21 levels in EoE are 1) possible differences in disease severity between patients in the two studies, 2) differences in assay sensitivity in detecting miR-21 and 3) differences in control patients between the two studies. Notably, for the serum analysis, the Lu *et al.* study compared circulating miR-21 in EoE patients to healthy atopic patients. In contrast, our controls in the EoE study were patients with normal histology of the upper GI tract who underwent EGD for non-specific reasons, but were not enriched for atopy. To better understand the relationship between atopy, allergic inflammatory disease and miR-21, we analyzed serum miR-21 levels in pediatric patients recruited into a study on the development of asthma. The patients were recruited as infants, based on a diagnosis of dermatitis, and subsequently characterized for atopic status and the development of asthma (Tepper *et al.*, 2008). Atopic status was defined as the presence of specific IgE to at least 1 out of 10 allergens tested. We found that in a randomly selected set of 16 patients, tested at 5 years of age, serum miR-21 increased in the asthma patients by 4-fold, with a *p* value of 0.018 (Figure 42A). MiR-22 showed no increase in patients with asthma (Figure 42A). In our selected patients, 8 out of 16 patients were atopic, however atopy was randomly distributed between asthmatic and non-asthmatic patients. While asthmatic patients had on average a two-fold increase in serum IgE over non-asthmatic patients (130 IU/ml versus 60 IU/ml), the

difference in average IgE level was not significant ( $p=0.24$ ). MiR-21 levels did not associate positively or negatively with atopy in this set of patients (Figure 42B). Thus, serum miR-21 is a novel biomarker for asthma, independent of atopy. Further research will be required to determine if serum miR-21 levels are a useful predictor of the development of asthma, EoE or other allergic inflammatory diseases.



**Figure 42: Increased miR-21 in the sera of pediatric patients with asthma.** (A) Quantitative PCR (QPCR) analysis of miR-21 and miR-22 expression in serum samples prepared from 5 year old patients presenting at 1 year of age with dermatitis and then monitored for allergy symptoms and the development of asthma. Relative miR expression with U6 as control miR. (B) Pearson correlation analysis between IgE levels and miR21 levels (fold change from average non-asthmatic control). N = 16 total patients, 8 with asthma and 8 without asthma. \* $p<0.05$  (two-tailed Student's t-test) (error bars, s.e.m.)

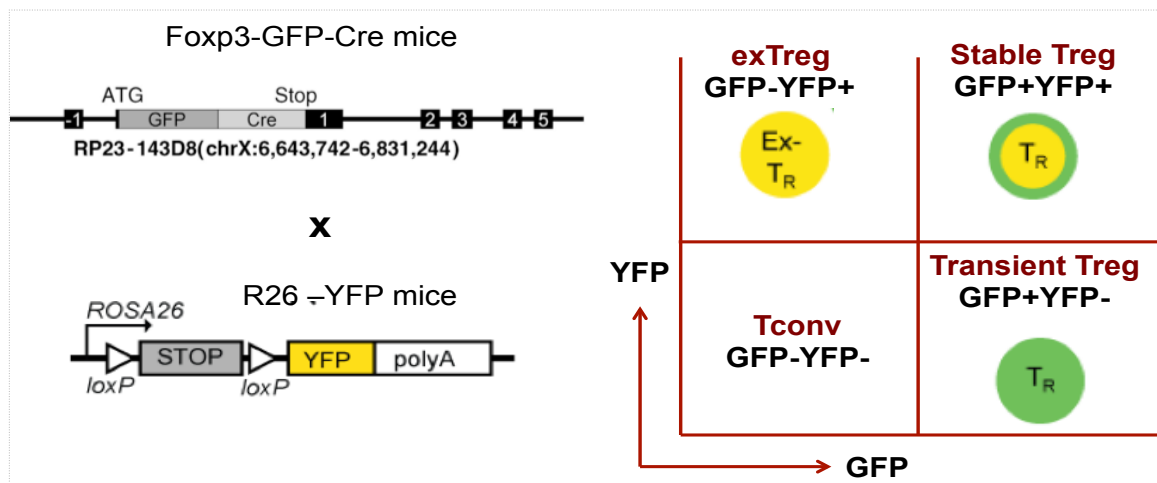
## **Part IV: Role of Bcl6 in Treg lineage stability in the context of the Th2 inflammatory disease in Bcl6-deficient mice**

### **Generation of dual reporter mice to assess stability of Foxp3 expression of Bcl6-deficient Tregs *in vivo***

Despite the central role of Tregs in maintaining immune tolerance, less is known about stability of the Treg phenotype *in vivo*. Most Tregs maintain high Foxp3 levels following adoptive transfer in a non-pathogenic setting (Floess *et al.*, 2007; Gavin *et al.*, 2007). However, recent reports employing Foxp3 reporter mice reveal that Foxp3 expression gets de-stabilized in inflammatory settings and these Tregs, termed “exTregs”, acquire cytokine production capabilities of effector T cells (Zhou *et al.*, 2009c). CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>+</sup> Tregs can convert to Bcl6-expressing Tfh cells in the mouse peyer’s patches (Tsuji *et al.*, 2009). Recently, TGFβ and retinoic acid present in the gut was shown to induce miR-10a, that targets Bcl6, thus maintaining Treg stability and preventing Treg conversion to Tfh cells (Takahashi *et al.*, 2012). Although our gene expression profiling of Bcl6-deficient Tregs did not show any reduction or loss of classical Treg gene signature, Bcl6-deficient Tregs exhibited a strong Th2-bias. One possible explanation is that the hyper-Th2 inflammation in Bcl6-deficient mice leads to destabilization of the Treg phenotype and reprogramming of the cells to Th2 fate. Therefore to perform genetic lineage tracing of Bcl6-deficient Foxp3<sup>+</sup> Tregs in the context of the hyper-Th2 inflammation *in vivo*, we crossed Bcl6-deficient mice with Foxp3-GFP-Cre x R26-YFP mice (designated as RFC strain). In the RFC mice, the Foxp3-GFP-Cre<sup>+</sup> Tregs excise the loxP-flanked stop codon



allowing for constitutive YFP expression from the R26 promoter, thus permanently labeling the Foxp3<sup>+</sup> T cells and their progeny (Zhou *et al.*, 2009c). This dual reporter mouse strain enables effective tracing of Foxp3 induction and down-regulation concurrently by two-color flow analysis of GFP and YFP expression (Figure 43). Due to a delay between Foxp3 transcription (GFP expression) and excision of the loxP-flanked stop cassette in the R26-YFP transgene by Cre, the GFP<sup>+</sup>YFP<sup>lo-neg</sup> population represents cells that recently up-regulated Foxp3 expression, GFP<sup>+</sup>YFP<sup>+</sup> cells represents the stable Tregs and the GFP<sup>-</sup>YFP<sup>+</sup> Treg population represents the exTregs. Monitoring the percentages of stable Tregs and exTregs in the peripheral lymphoid organs and the inflamed heart and lungs in Bcl6-deficient mice (KO RFC) relative to the wild-type counterparts (WT RFC) will provide useful insights into the impact of the Th2 inflammatory environment on Bcl6-deficient Treg stability as well as the contribution of these exTregs to the Th2 disease.

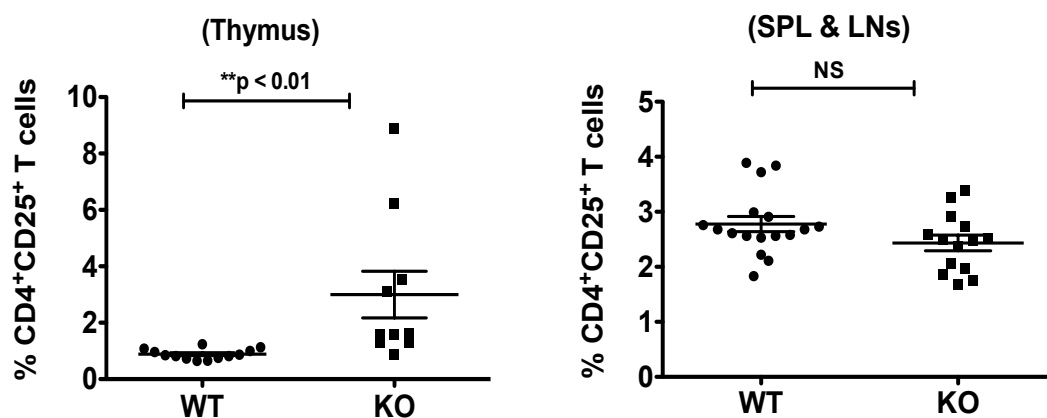


**Figure 43: Schematic for isolating stable Tregs and exTregs using the Foxp3 lineage tracing mouse model.**

Stable Tregs (GFP<sup>+</sup>YFP<sup>+</sup>) and exTregs (GFP<sup>-</sup>YFP<sup>+</sup>) populations from WT and KO RFC mice will be assayed by flow analysis of YFP/GFP expression following surface staining of thymus and (SPL+LN) CD4<sup>+</sup>CD25<sup>+</sup> T cells. Cell populations will also be sorted for characterization of phenotype and function.

### Bcl6-deficient mice exhibit increased exTregs in the periphery

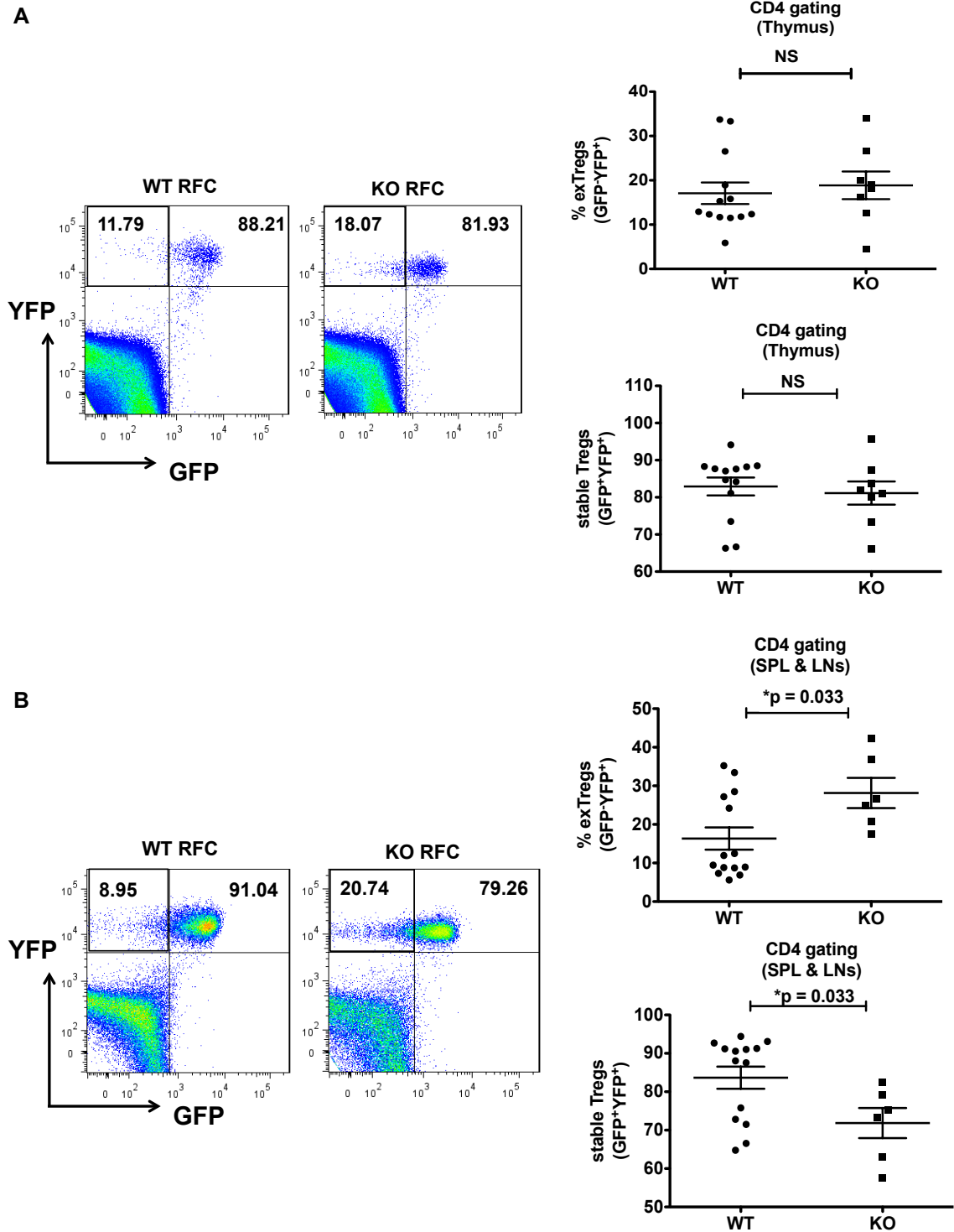
Bcl6-deficient mice on the Foxp3-GFP-Cre x R26-YFP background (KO RFC) developed myocarditis and lung vasculitis at an accelerated rate compared to the germ-line Bcl6-deficient mice. While the germ-line Bcl6-deficient mice die about 6-12 weeks of age, most of the KO RFC mice die by 3-5 weeks of age. Histology of the heart and lungs demonstrated severe inflammation in the heart and lungs (data not shown) compared to germ-line Bcl6-deficient mice that can explain their accelerated death rate. We predict that the increased inflammatory response in this strain is likely due to effects from the NOD background of the Foxp3-GFP-Cre mice or a genetic drift effect. ExTregs have been shown to down-regulate expression of the classical Treg surface marker, CD25 (Zhou *et al.*, 2009c). However, we noted either increased or intact percentages of CD4<sup>+</sup>CD25<sup>+</sup> T cells in the thymus and (SPL+LN) preparations (Figure 44).



**Figure 44: Increased percentages of CD4<sup>+</sup>CD25<sup>+</sup> T cells in the thymus and similar percentages in the periphery of WT and KO RFC mice.**

Cell preparations from wild-type (WT) and Bcl6-deficient (KO) RFC mice were stained for classical Treg surface markers – CD4 and CD25 and the assessed by flow analysis. N=10-15 mice per group. \*\*p<0.01 (two-tailed Student's t-test) error bars = s.e.m.)

About 10% of peripheral CD4<sup>+</sup> T cells are reported to be GFP<sup>+</sup>YFP<sup>+</sup> (stable Tregs) and about 15-20% of the peripheral YFP<sup>+</sup> cells can convert to GFP<sup>-</sup>YFP<sup>+</sup> exTregs (Zhou *et al.*, 2009c). Analysis of the thymus in the WT and KO RFC mice demonstrated a comparable percentage of both stable Tregs and exTregs, thereby suggesting that the KO Tregs are not intrinsically biased towards losing Foxp3 expression (Figure 45A). On the other hand, analysis of these populations in the peripheral lymphoid organs (spleen and lymph nodes) demonstrated a significant increase in the percentage of exTregs and a corresponding decrease in stable Treg numbers in the KO RFC mice relative to WT RFC (Figure 45B). This indicated that the Th2 inflammatory environment in the Bcl6-deficient mice de-stabilizes Tregs and favors the loss of Foxp3 expression. While we did not detect any obvious difference in the percentages of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in the peripheral lymphoid organs of WT and Bcl6-deficient mice on the Foxp3-gfp background (as shown in Figure 5), the heightened proliferative potential of the Bcl6-deficient Tregs (as shown in Figure 8) may contribute to their high turnover rate, thus necessitating the use of dual reporters to track their stability *in vivo*. The increased inflammatory background in the KO RFC may accelerate the down-regulation of Foxp3 in the Bcl6-deficient Tregs. While the exact reason for the increased exTreg generation on the KO RFC background remains unclear, this data suggests that the absence of Bcl6 exacerbates the instability of Foxp3 under the inflammatory setting.



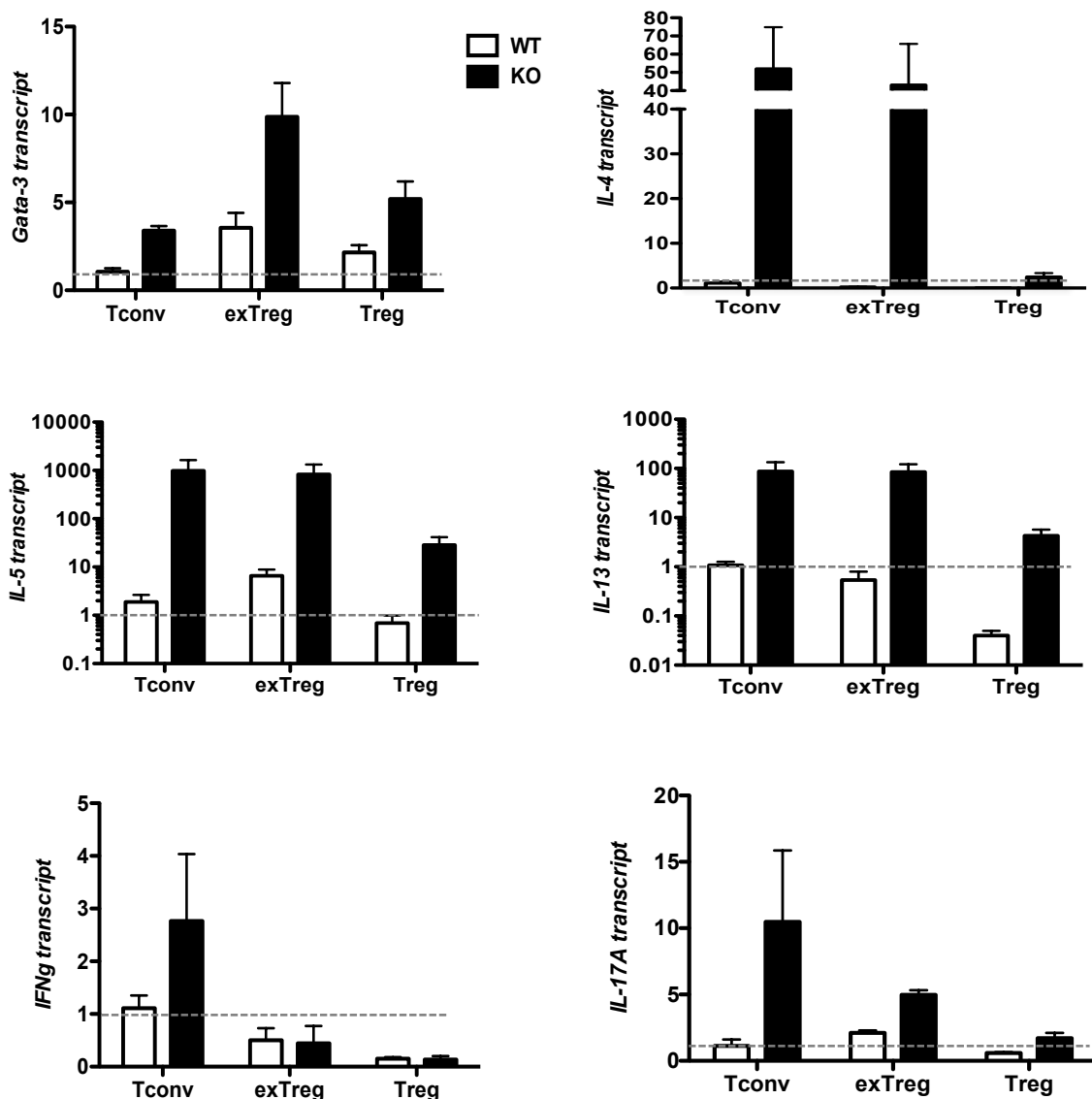
**Figure 45: Increased percentages of exTregs and reduced stable Tregs in peripheral lymphoid organs of KO RFC mice.**

Cell preparations from wild-type (WT) and Bcl6-deficient (KO) RFC mice from the thymus **(A)** and (SPL+LN) **(B)** were stained for CD4 and assessed by flow analysis for YFP/GFP. Cells were gated as CD4<sup>+</sup> T cells to assess percentages of stable (GFP<sup>+</sup>YFP<sup>+</sup>) and exTregs (GFP<sup>-</sup>YFP<sup>+</sup>) populations. N=10-15 mice per group. \*p<0.05 (two-tailed Student's t-test) error bars = s.e.m.

## **GFP<sup>+</sup>YFP<sup>+</sup> stable Tregs and GFP<sup>-</sup>YFP<sup>+</sup> exTregs exhibit a strong Th2-bias in the absence of Bcl6**

ExTreg (GFP<sup>-</sup>YFP<sup>+</sup>) cells have been reported to exhibit an activated-memory phenotype with reduced (CD25, GITR) or heterogeneous (CTLA-4, FR4, CD103) expression of Treg signature genes, heterogeneous CD62L, high CD44 and also acquire effector cytokine-producing ability (IFN $\gamma$  or IL17A) based on the microenvironment (Zhou *et al.*, 2009c). In order to assess the effect of the Th2 inflammatory environment on the Treg and exTreg phenotype in Bcl6-deficient mice, we sorted CD25<sup>-</sup> Tconv cells, GFP<sup>+</sup>YFP<sup>+</sup> stable Tregs and GFP<sup>-</sup>YFP<sup>+</sup> exTregs from WT and KO RFC mice, activated them *in vitro* and assessed their gene expression and cytokine secretion signature. Consistent with the well-demonstrated role for Bcl6 in inhibiting Th2 differentiation (Kusam *et al.*, 2003), we noted increased expression of Th2 genes (*Gata3*, *Il4*, *Il5*, *Il13*) in KO Tconv cells, relative to WT (Figure 46). This translated into increased secretion of Th2 cytokines by KO Tconv cells as represented in Table 4. Although IL-17A expression was increased in KO Tconv cells, IFN $\gamma$  was not significantly different. Importantly, the stable Tregs from the KO RFC mice also exhibited a selective Th2-bias, consistent with our gene expression array data with Bcl6-deficient Foxp3gfp Tregs (Figure 46). Specifically Th2 genes were strongly elevated in KO Tregs, confirming that Bcl6 also represses Th2 gene expression in Treg cells. The overall levels of Th2 gene expression in the Treg fraction was reduced in KO Tregs compared to KO Tconv cells, consistent with a role of Foxp3 in limiting effector cytokine expression in Tregs. On the other hand, the KO exTregs

acquired a Th2-bias, suggesting that the exTregs were remodeled in the context of the Th2 inflammatory environment in the RFC mice (Figure 46). Although *Il17a* was increased in KO exTregs, the induction was not as potent as the Th2 genes, while *Ifng* remained unchanged. Interestingly, the induction of Th2 genes in the KO exTregs was comparable to the levels in the KO Tconv cells, indicating that the Tregs that lose Foxp3 under inflammatory contexts acquire effector cytokine-producing abilities similar to effector T cells. Thus the Th2-biased exTregs in KO RFC mice may contribute to the exacerbation of the inflammatory Th2 responses and thus the heart and lung-specific pathology in Bcl6-deficient mice. Thus, while the increased Th2 gene signature in the KO stable Tregs strongly confirms an intrinsic Th2-bias of Tregs in the absence of Bcl6, the potent Th2 gene induction in the KO exTregs suggests that the Th2 inflammatory disease strongly favors loss of Foxp3 expression and remodeling of Tregs to Th2 effectors. Thus, these studies suggest that the Th2-biased stable Tregs and environmentally re-programmed exTregs can contribute to the increased Th2 polarization and exacerbated inflammatory disease in the Bcl6-deficient mice.



**Figure 46: Potent Th2-bias of Tconv, stable Tregs and exTregs derived from KO RFC mice.**

Tconv ( $CD4^+CD25^-$ ), stable Tregs ( $CD4^+CD25^+GFP^+YFP^+$ ) and exTregs ( $CD4^+CD25^+GFP^-YFP^+$ ) populations from spleen and lymph node preparations of wild-type (WT) and Bcl6-deficient (KO) RFC mice were FACS-sorted and then stimulated with anti-CD3 and anti-CD28 for 12-16 hours for gene expression analysis. Data represent average gene expression normalized using tubb5 as endogenous control. N= 5 WT and 4 KO RFC mice.

Mice Cytokine	WT RFC			KO RFC		
(pg/ml)	Tconv GFP <sup>-</sup> YFP <sup>-</sup>	ExTreg GFP <sup>-</sup> YFP <sup>+</sup>	Stable Treg GFP <sup>+</sup> YFP <sup>+</sup>	Tconv GFP <sup>-</sup> YFP <sup>-</sup>	ExTreg GFP <sup>-</sup> YFP <sup>+</sup>	Stable Treg GFP <sup>+</sup> YFP <sup>+</sup>
IL-4	229 ± 122	5 ± 2	3 ± 1	9713 ± 3614	7498 ± 3650	895 ± 424
IL-5	27 ± 5	23 ± 1	6 ± 1	6054 ± 2597	3929 ± 1692	904 ± 196
IL-13	14 ± 4	N.D.	N.D.	4663 ± 2815	2460 ± 1220	310 ± 128
IL-10	114 ± 14	388 ± 116	271 ± 31	1822 ± 1279	3626 ± 1470	3670 ± 353
IFN $\gamma$	978 ± 2	108 ± 34	71 ± 28	1348 ± 609	120 ± 28	150 ± 86
IL-17	219 ± 58	107 ± 33	65 ± 1	1680 ± 521	1094 ± 183	880 ± 67

**Table 4: Cytokine levels in Tconv, exTreg and stable Treg subsets from WT and KO RFC mice.**

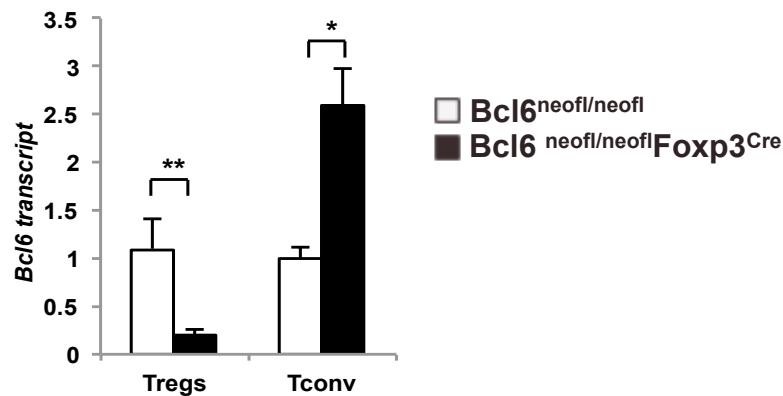
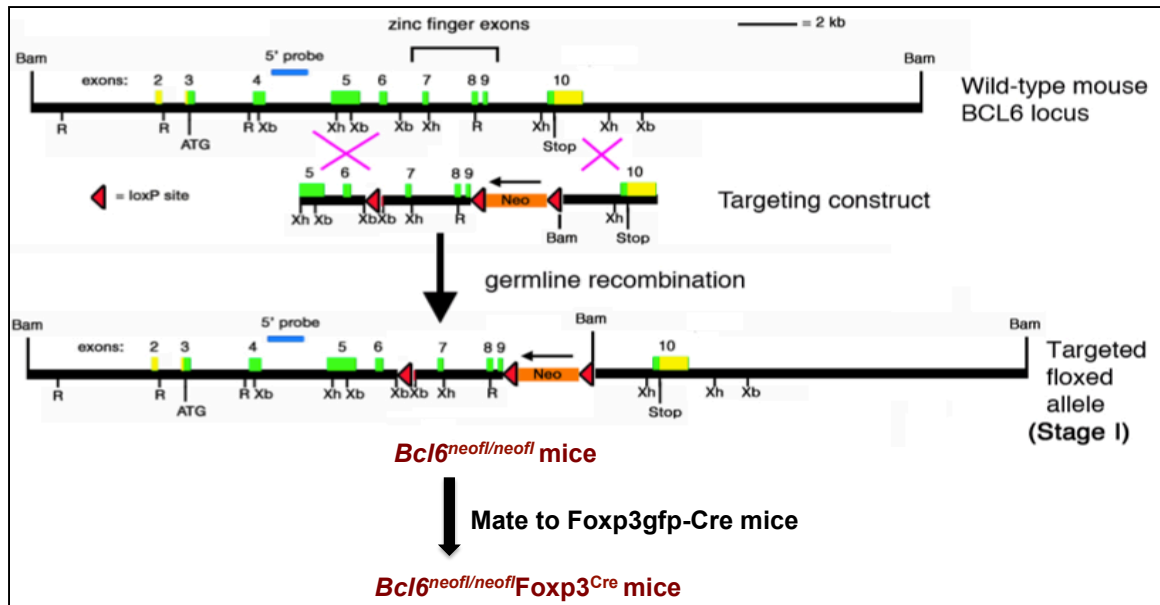
Supernatants harvested from overnight-activated FACS sorted Tconv cells (CD4<sup>+</sup>CD25<sup>-</sup>), exTregs (CD4<sup>+</sup>CD25<sup>+</sup>GFP<sup>-</sup>YFP<sup>+</sup>) and stable Tregs (CD4<sup>+</sup>CD25<sup>+</sup>GFP<sup>+</sup>YFP<sup>+</sup>), assessed by multiplex analysis. Data represented is an average from 2 WT and 3 KO RFC mice.



## **Part V: Characterization of Treg-specific Bcl6 conditional knockout mice**

### **Generation of Treg-specific Bcl6 conditional mutant ( $Bcl6^{neofl/neofl}Foxp3^{Cre}$ ) mice (Stage I)**

To test whether the promotion of Th2 inflammation by the Bcl6-deficient Tregs was an intrinsic effect of loss of Bcl6 within the Treg lineage, we generated Treg specific Bcl6 conditional mutant mice. A conditional KO allele for Bcl6, where the zinc finger-encoding exons of Bcl6 were flanked by loxP sites ( $Bcl6^{fl/fl}$ ), was constructed and used to target ES cells (Figure 47). After generation of chimeric mice, the  $Bcl6^{fl/fl}$  allele was transmitted to offspring mice (Stage I). These Stage I mice carry the  $Bcl6^{fl/fl}$  allele containing the neomycin gene flanked by loxP sites, hence referred as  $Bcl6^{neo/fl/neo/fl}$  mice (Stage I). These mice were mated to Foxp3-gfpCre mice to generate mice with Treg-specific deletion of Bcl6 ( $Bcl6^{neofl/neofl}Foxp3^{Cre}$  mice).  $Bcl6^{neofl/neofl}Foxp3^{Cre}$  mice exhibit almost 90% loss of Bcl6 expression in Treg cells, whereas Bcl6 expression was increased in conventional CD4 T cells (Tconv) from the conditional mutant mice, indicating specific loss of Bcl6 in the Treg lineage (Figure 47).



**Figure 47: Treg-specific deletion of Bcl6 (Stage I).**

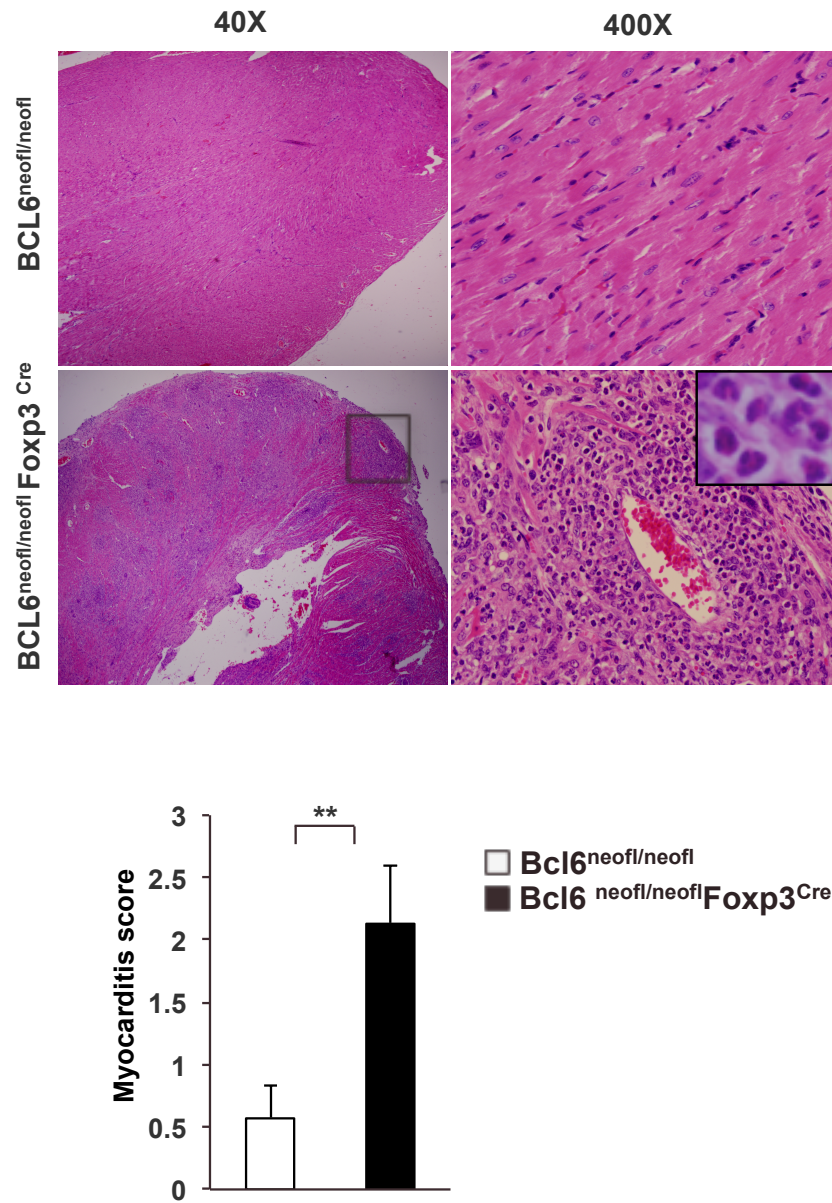
(Top) Targeting construct for the generation of *Bcl6*<sup>neofl/neofl</sup> mice. These mice were mated to Foxp3-gfpCre mice to generate *Bcl6*<sup>neofl/neofl</sup>Foxp3<sup>Cre</sup> mice.

(Bottom) QPCR analysis of *Bcl6* transcript between *Bcl6*<sup>neofl/neofl</sup>Foxp3<sup>Cre</sup> (black bars) and *Bcl6*<sup>neofl/neofl</sup> (white bars) in FACS-sorted CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> (Tregs) and MACS-isolated CD4<sup>+</sup>CD25<sup>-</sup> (Tconv) cells, following activation for 16 hrs with anti-CD3 and anti-CD28. Relative gene expression normalized to *tubb5*. N=3 (*Bcl6*<sup>neofl/neofl</sup>) and n=6 (*Bcl6*<sup>neofl/neofl</sup>Foxp3<sup>Cre</sup>) groups, respectively.

\*p<0.05, \*\*p<0.01, \*\*\*p<0.001 (two-tailed Student's t-test) (error bars, s.e.m.)

## **Heart-specific inflammatory disease in the $Bcl6^{neofl/neofl}Foxp3^{cre}$ (Stage I) mice, similar to germ-line $Bcl6$ knockout mouse**

Strikingly, deletion of  $Bcl6$  expression specifically in the Treg lineage led to a high rate of spontaneous and severe myocarditis (Figure 48). Like the myocarditis observed in germ-line  $Bcl6$ -deficient mice, the myocarditis in  $Bcl6^{neofl/neofl}Foxp3^{cre}$  mice was associated with eosinophils (Fig 48, inset) and was observed at a high frequency (10/16 mice = 63%). Further investigation of the Stage I  $Bcl6^{neofl/neofl}$  mice revealed that these mice also develop myocarditis, but at a lower frequency and with less severity. While the  $Bcl6^{neofl/neofl}Foxp3^{cre}$  mice exhibit a 90% loss of  $Bcl6$  expression in the Treg lineage, we suspect that the *neo* gene down-regulates  $Bcl6$  expression to a significant but lesser extent in other lineages. The  $Bcl6^{neofl}$  gene may represent a “hypomorphic” *Bcl6* allele, with expression of functional  $Bcl6$  protein but overall decreased  $Bcl6$  expression compared to wild-type. Thus, the severe myocarditis in  $Bcl6^{neofl/neofl}Foxp3^{cre}$  mice may be a combination of decreased  $Bcl6$  expression in lymphoid and myeloid cells, with the Treg defects from full loss of  $Bcl6$  leading to failure to control the Th2 pathology, in a manner similar to germ-line  $Bcl6$ -deficient mice.

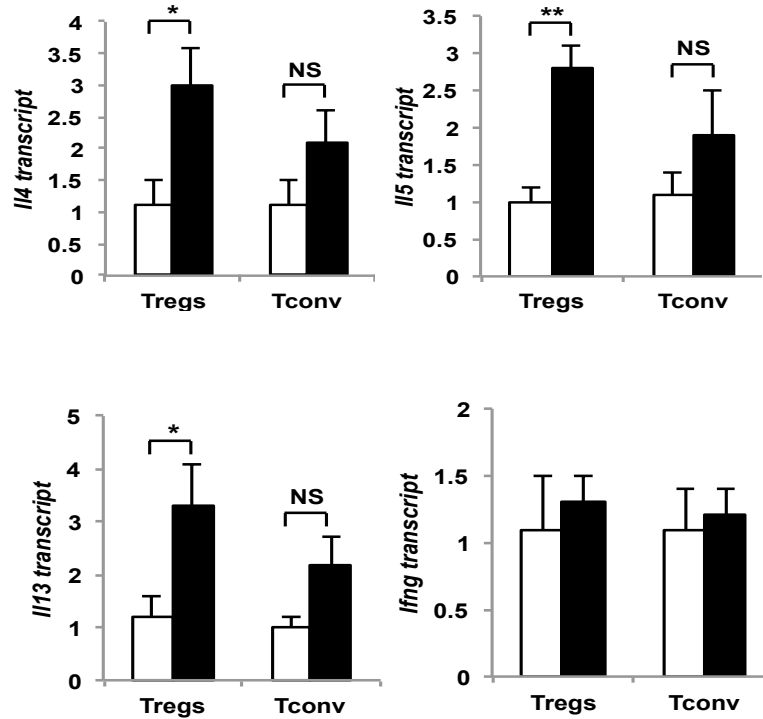


**Figure 48: Treg-specific Bcl6 conditional mutant mice (Stage I Bcl6<sup>neofl/neofl</sup> Foxp3<sup>Cre</sup>) develop spontaneous myocarditis.**

(Left) Heart histopathology assessed by H & E staining of paraffin-embedded sections for Bcl6<sup>fl/fl</sup> (top panel) and Bcl6<sup>fl/fl</sup> Foxp3<sup>Cre</sup> (bottom panel). Original magnification, 40X (left) and 400X (right). Inset in 400X in Bcl6<sup>fl/fl</sup> Foxp3<sup>Cre</sup> shows high power view of eosinophils. (Right) Heart sections from Bcl6<sup>fl/fl</sup> Foxp3<sup>Cre</sup> (black bars) and Bcl6<sup>fl/fl</sup> (white bars) mice were scored in a blinded-fashion on a scale of 0-4 to assess the severity of myocarditis. N=14 (Bcl6<sup>fl/fl</sup>) and N=16 (Bcl6<sup>fl/fl</sup> Foxp3<sup>Cre</sup>) groups. (\*\*p =0.0096)

### **Intrinsic regulation of Th2 gene expression in Tregs by Bcl6**

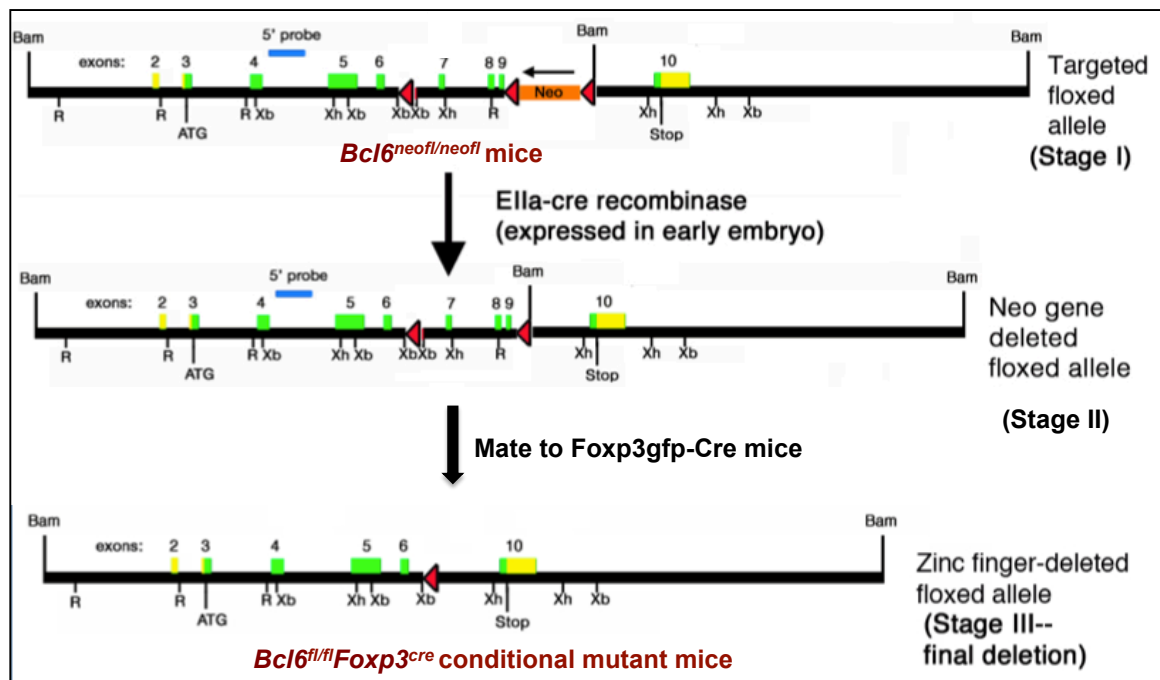
To obtain FoxP3<sup>+</sup> Tregs from control Bcl6<sup>neofl/neofl</sup> mice, we bred the FoxP3-gfp allele used in our earlier studies to this strain. Foxp3<sup>+</sup> Tregs from Bcl6<sup>neofl/neofl</sup>Foxp3<sup>cre</sup> mice displayed a significant increase in Th2 cytokines (*Il4*, *Il5*, *Il13*) but not an increase in the Th1 cytokine, *Ifng* (Figure 49), when compared to FoxP3<sup>+</sup> Tregs from Bcl6<sup>neofl/neofl</sup> mice. Our results were consistent with our microarray analysis of Tregs from germline Bcl6-deficient mice. Tconv from Bcl6<sup>neofl/neofl</sup>Foxp3<sup>cre</sup> mice did not show a significant increase in Th2 cytokines (Figure 49), showing specific effects of loss of Bcl6 in the Treg lineage. These results from the Stage I Bcl6<sup>neofl/neofl</sup>Foxp3<sup>cre</sup> mice strongly support the idea that Bcl6-deficient Tregs have an intrinsic defect that results in increased Th2 cytokine expression and further allows them to promote, instead of suppress Th2-type inflammation.



**Figure 49: Bcl6 intrinsically represses Th2 gene expression in Treg cells.** Quantitative PCR analysis of Th2 genes (*Il4*, *Il5*, *Il13*) and *Ifng* transcripts between Bcl6<sup>neofl/neofl</sup> Foxp3<sup>cre</sup> (black bars) and Bcl6<sup>neofl/neofl</sup> (white bars) FACS-sorted CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> (Tregs) and MACS-isolated CD4<sup>+</sup>CD25<sup>-</sup> (Tconv) cells following activation for 16 hrs with anti-CD3 and anti-CD28. Relative gene expression is shown, where expression is normalized to *tubb5*. Data are average expression from n=3 (Bcl6<sup>neofl/neofl</sup>) and n=6 (Bcl6<sup>neofl/neofl</sup> Foxp3<sup>cre</sup>) groups, respectively.

## Generation of Bcl6 conditional mutant ( $Bcl6^{fl/fl}Foxp3^{cre}$ ) mice (Stages II and III)

The next step was to assess the ability of Bcl6-deficient Tregs from the conditional mutant mice to control Th2 inflammatory responses *in vivo*. While we do note an intrinsic increase in Th2 cytokine expression from Tregs derived from the  $Bcl6^{neofl/neofl}Foxp3^{cre}$  mice, these mice still retain the neo gene that affects Bcl6 expression in other lineages and develop a Th2-biased inflammatory pathology in their hearts. Thus, to definitively assess control of Th2 responses by Bcl6-deficient Tregs, we generated the neo-deleted  $Bcl6^{fl/fl}$  (Stage II) mice by mating  $Bcl6^{neofl/neofl}$  mice to mice expressing E1a-Cre (Figure 50). These neo-deleted  $Bcl6^{fl/fl}$  (Stage II) mice were then mated to Foxp3gfp Cre to generate the final Treg-specific conditional mutant ( $Bcl6^{fl/fl}Foxp3^{cre}$ ) Stage III mice (Figure 50).



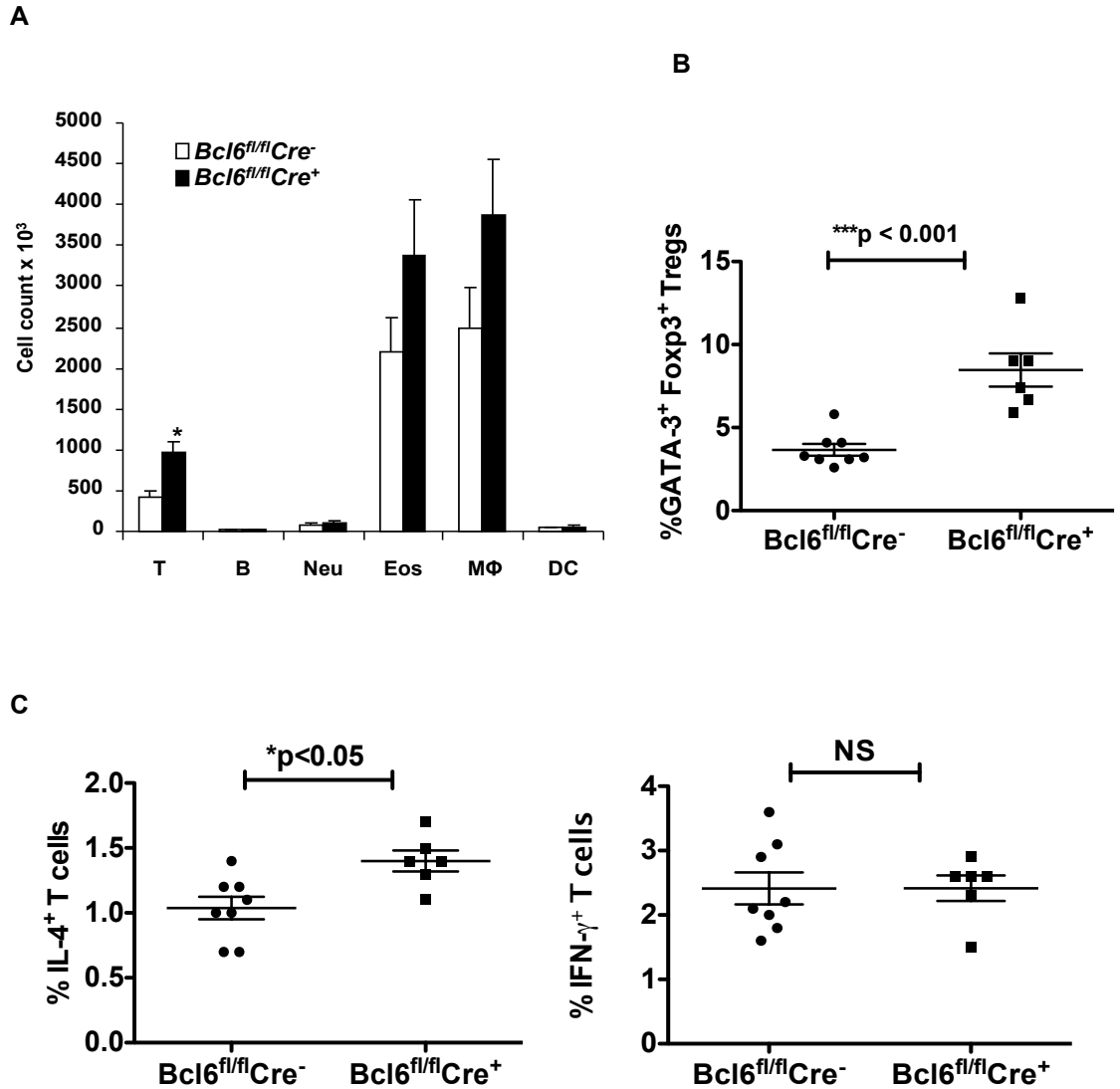
**Figure 50: Treg-specific deletion of Bcl6 (Stages II & III).**

$Bcl6^{neofl/neofl}$  mice (Stage I) were mated to mice expressing E1a-cre recombinase. The resultant neo-gene deleted Bcl6 floxed mice (Stage II) were mated to Foxp3gfp Cre mice to generate the final Treg-specific Bcl6 conditional mutant ( $Bcl6^{fl/fl}Foxp3^{cre}$ ) mice (Stage III).

**Tregs derived from Bcl6 conditional mutant ( $Bcl6^{fl/fl}Foxp3^{cre}$ ) mice acquire a Th2-bias and fail to suppress Th2 allergic airway inflammation *in vivo***

The final Stage III Treg-specific Bcl6 conditional mutant ( $Bcl6^{fl/fl}Cre^{+}$ ) mice appear grossly normal and in preliminary assessments showed no development of myocarditis (not shown). To further characterize these mice,  $Bcl6^{fl/fl}Cre^{+}$  mice and their littermate controls ( $Bcl6^{fl/fl}Cre^{-}$ ) mice were tested for induction of allergic airway inflammation using the same sensitization and challenge protocol as shown in Figure 12. We noted that the  $Bcl6^{fl/fl}Cre^{+}$  mice exhibited increased inflammatory cell accumulation in the lung airways, particularly T cells, as assessed from flow analysis of the bronchoalveolar lavage (BAL) fluid (Figure 51A). The  $Bcl6^{fl/fl}Cre^{+}$  Tregs demonstrated a significant increase in expression of the Th2 transcription factor, GATA-3 (Figure 51B). This is consistent with a Th2-biased phenotype noted with germline Bcl6-deficient Tregs. Interestingly, these  $Bcl6^{fl/fl}Cre^{+}$  Tregs also failed to suppress Th2 allergic responses as shown by increased percentages of IL-4<sup>+</sup> T cells in the periphery for  $Bcl6^{fl/fl}Cre^{+}$  mice, compared to controls, while the percentage of IFN- $\gamma$ <sup>+</sup> T cells remained unchanged in the two groups (Figure 51C). Thus, these results validate our results with Tregs derived from the germline Bcl6-deficient mice and confirm an intrinsic role for Bcl6 in Treg control of Th2 inflammatory responses *in vivo*.



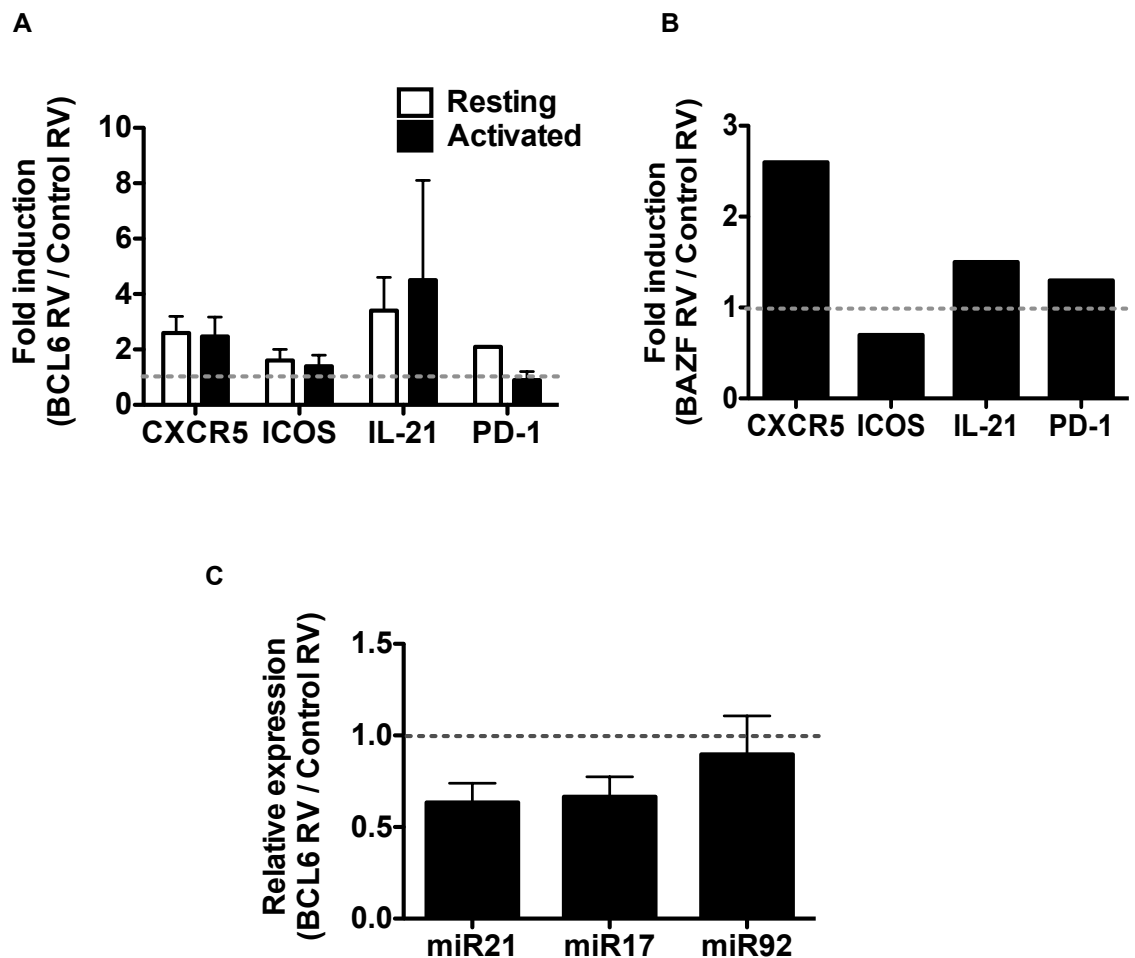


**Figure 51: Bcl6 conditional mutant ( $Bcl6^{fl/fl}Foxp3^{Cre}$ ) Tregs fail to suppress Th2 allergic airway inflammation *in vivo*.**

(A) Flow analysis of different cell types (T cells, B cells, Eos, Macrophages and DCs) from the BAL fluid from sensitized and challenged Treg-specific Bcl6 conditional mutant ( $Bcl6^{fl/fl}Cre^{+}$ ) mice and their littermate controls ( $Bcl6^{fl/fl}Cre^{-}$ ) mice. (B) ICS depicting percentage of GATA-3<sup>+</sup>Foxp3<sup>+</sup> Tregs derived from the spleens of Treg-specific Bcl6 conditional mutant ( $Bcl6^{fl/fl}Cre^{+}$ ) mice and their littermate controls ( $Bcl6^{fl/fl}Cre^{-}$ ) following activation with PMA and Ionomycin. (C) ICS depicting percentage of IL-4<sup>+</sup> and IFN- $\gamma$ <sup>+</sup> T cells derived from the spleens of Treg-specific Bcl6 conditional mutant ( $Bcl6^{fl/fl}Cre^{+}$ ) mice and their littermate controls ( $Bcl6^{fl/fl}Cre^{-}$ ) following activation with PMA and Ionomycin. N=6 ( $Bcl6^{fl/fl}Cre^{+}$ ) mice and 8 ( $Bcl6^{fl/fl}Cre^{-}$ ) mice, respectively. \*p<0.05 (two-tailed Student's t-test) (error bars, s.e.m.)

## **Bcl6 and BAZF (Bcl6b) induce differentiation of naïve CD4<sup>+</sup> T cells to Tfh fate *in vitro***

Bcl6 is the master transcription factor for T follicular helper cells, subset of T cells that help germinal center B cells in the production of high affinity plasma cells and memory B cells (Johnston *et al.*, 2009; Nurieva *et al.*, 2009; Yu *et al.*, 2009). Bcl6-deficient mice fail to form Tfh cells and germinal centers. Importantly, Bcl6 over-expression alone is sufficient to induce Tfh differentiation of naïve CD4<sup>+</sup> T cells and as shown in Figure 52A. We note induction of the Tfh signature genes (CXCR5, ICOS, IL-21, PD-1) in Bcl6 RV transduced T cells under both resting and activated conditions. Expression of a known Bcl6 homologue, BAZF (Bcl6b) is increased to the same extent as Bcl6 in antigen-primed Tfh cells (Fazilleau *et al.*, 2009; Pelletier *et al.*, 2010). This prompted us to test the functional relevance of BAZF in the Tfh differentiation program. We note that similar to retroviral transduction of Bcl6, BAZF RV transduced T cells also demonstrated an increased in Tfh genes (CXCR5, IL-21, PD-1) relative to control RV (Figure 52B). Thus, this suggests that BAZF may also contribute to the Tfh developmental program. Being a well-characterized transcriptional repressor, Bcl6 probably positively directs Tfh differentiation by repressing miRNAs that target key Tfh genes (Yu *et al.*, 2009). Bcl6 represses miR-17-92 that targets the chemokine receptor, CXCR5. MiR-21 is one of the most strongly down-regulated miRNA by Bcl6 in T cells as shown (Figure 52C), although its functional relevance to Tfh differentiation is unknown.



**Figure 52: Bcl6 and Bazf direct Tfh differentiation *in vitro*.**

**(A)** Quantitative PCR analysis of Tfh genes (*Cxcr5*, *Icos*, *Il21*, *Pd1*) in Bcl6 RV transduced CD4 T cells, with their expression in control RV transduced cells set to 1. Fold induction (Bcl6 RV/Control RV) of Tfh genes reported in FACS-sorted RV transduced resting cells (white bars) and following activation for 6-12 hrs with anti-CD3 and anti-CD28 (black bars). Data are averaged from at least 3 independent experiments.

**(B)** Quantitative PCR analysis of Tfh genes (*Cxcr5*, *Icos*, *Il21*, *Pd1*) in Bazf RV transduced CD4 T cells, with their expression in control RV transduced cells set to 1, following activation for 6-12 hrs with anti-CD3 and anti-CD28.

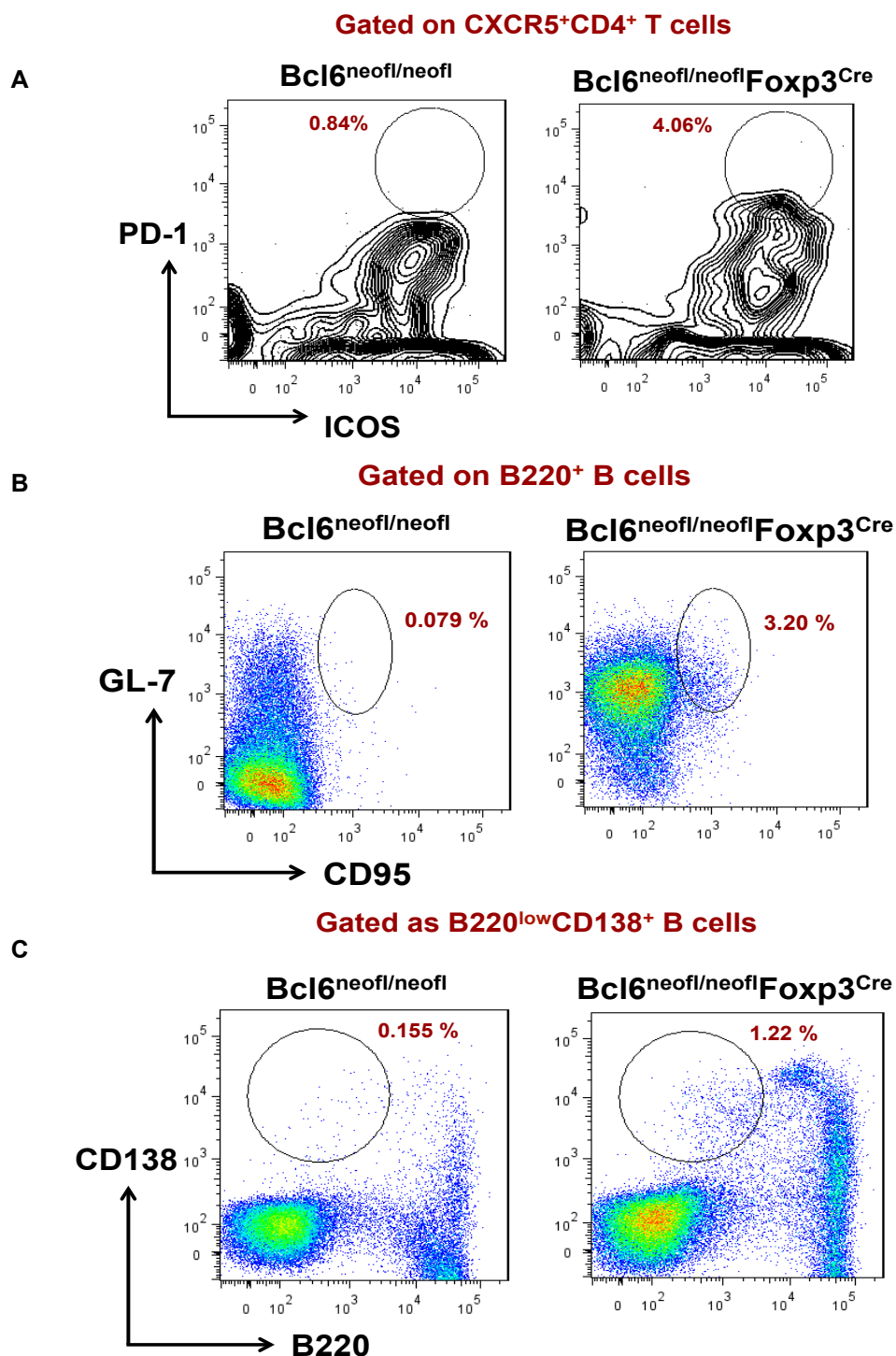
**(C)** Quantitative PCR analysis of miRNAs (21, 17, 92) in Bcl6 RV transduced CD4 T cells, with their expression in control RV transduced cells set to 1. Fold repression (Bcl6 RV/Control RV) of miRNAs reported following activation of RV transduced cells for 6-12 hrs with anti-CD3 and anti-CD28. Data are averaged from at least 3 independent experiments.

## **Bcl6 specifies development of T follicular regulatory cells, specialized in control of germinal center response**

T follicular regulatory cells (Tfr) are a sub-population of Tregs that co-express Bcl6 and CXCR5 and localize to the germinal centers in mice and humans (Chung *et al.*, 2011; Linterman *et al.*, 2011). These cells up-regulate the hallmark Tfh markers (CXCR5, BTLA, PD-1, ICOS) in a Bcl6-dependent manner. Thus, Bcl6-deficient CD4<sup>+</sup>CD25<sup>hi</sup> Tregs exhibit decreased expression of Cxcr5, Pdccl1 and Btla (Chung *et al.*, 2011). Similar decreases in Tfh markers was also noted in our Bcl6-deficient CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs derived from Foxp3gfp mice (data not shown). Foxp3-deficient scurfy mice lack Tfr cells and exhibit moderate increases in Tfh cell numbers, but marked increase in GC B cells, indicating that these cells are indispensable for regulating the germinal center B cell response. Since Bcl6-conditional mutant mouse strains were unavailable, earlier reports by Linterman *et al.* (Linterman *et al.*, 2011) and Chung *et al.* (Chung *et al.*, 2011) relied on indirect methods to assess the functional relevance of Tfr cells – fetal liver chimeras of Bcl6<sup>+/+</sup>: Bcl6<sup>-/-</sup> and Sh2d1a<sup>-/-</sup> or Sh2d1a<sup>+/+</sup>: Foxp3DTR, or adoptive transfers of Bcl6-deficient and CXCR5-deficient Tregs to Tcrβ-deficient mice.

Thus, the Treg-specific Bcl6 conditional knockout mouse strain we have generated is ideal to define the functional implications of Tfr cells in regulating both the magnitude and quality of the germinal center reaction. We note that unimmunized Bcl6<sup>neofl/neofl</sup> Foxp3<sup>cre</sup> (Stage I) mice exhibit spontaneous increase in Tfh cells, GC B cells and plasma cells, compared to Bcl6<sup>neofl/neofl</sup> control mice

(Figure 53). Similar results were obtained comparing unimmunized Stage III Treg-specific Bcl6 conditional mutant ( $Bcl6^{fl/fl}Cre^{+}$ ) mice, relative to controls ( $Bcl6^{fl/fl}Cre^{-}$ ). This confirms that defects in Tfr cell development lead to uncontrolled Tfh and germinal center activity.



**Figure 53: Bcl6<sup>neofl/neofl</sup>Foxp3<sup>Cre</sup> mice have increased spontaneous Tfh, GC B and plasma cells.**

Spleen cell preparations from un-immunized Bcl6<sup>neofl/neofl</sup>Foxp3<sup>Cre</sup> and control Bcl6<sup>neofl/neofl</sup> (Cre negative) mice were stained for the indicated Tfh (CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup>PD-1<sup>+</sup>) (**A**), GC B (B220<sup>+</sup>GL-7<sup>+</sup>CD95<sup>+</sup>) (**B**) and plasma cell (B220<sup>low</sup>CD138<sup>+</sup>) (**C**) surface markers. N=2/group.

## DISCUSSION

### **Bcl6-deficient Treg cells exhibit a selective failure to suppress Th2 immune responses *in vivo***

In the past few years, Bcl6 has gained a great deal of attention as a master regulator of T follicular helper (Tfh) cells, and has been shown to inhibit differentiation towards Th1, Th2 and Th17 fates (Dent *et al.*, 1998a; Dent *et al.*, 1997; Johnston *et al.*, 2009; Kusam *et al.*, 2003; Nurieva *et al.*, 2009; Yu *et al.*, 2009). Lack of germinal centers and failure to mount protective secondary antibody responses are the key observations underlying its role as a master transcription factor for Tfh and GC B cells. In addition, the absence of Bcl6 manifests as a spontaneous and severe Th2-mediated inflammatory disease, affecting the heart and lungs, early in life. The inflammatory disease in Bcl6-deficient mice is complex and involves multiple cell types, however, the disease is completely dependent upon T cells (Table 3). While the Bcl6-deficient APC-derived pro-Th17 cytokines also contribute to the disease severity (Mondal *et al.*, 2010), the Th2 inflammatory response is dominant to the pathology. These observations underscore the well-characterized role for Bcl6 in inhibiting Th2 differentiation, but may also suggest that Th2 inflammatory responses increase in severity due to defective Treg responses in the absence of Bcl6. Very recent work has shown that Bcl6 is involved in the formation of a novel Foxp3<sup>+</sup> subset of follicular T helper cells referred as “Tfr” cells that regulate the germinal center response (Chung *et al.*, 2011; Linterman *et al.*, 2011). However, the function of Bcl6 in Treg-mediated control of inflammatory T cell responses has not been

studied, despite the organ-specific Th2 pathology in germ-line Bcl6-deficient mice. In this work, we have undertaken the first in-depth analysis of Bcl6-deficient Treg cells to gain insights into the regulation of this inflammation.

Our studies have employed Bcl6-deficient Tregs derived from Foxp3-gfp reporter mice that ensure isolation of highly purified CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs. In a previous report, Chung *et al* reported a dispensable role for Bcl6 in control of T cell responses by testing CD4<sup>+</sup>CD25<sup>hi</sup> Tregs *in vivo* (Chung *et al.*, 2011). However, we have observed that these CD4<sup>+</sup>CD25<sup>hi</sup> Treg preparations also include contaminating activated Foxp3<sup>-</sup> Th2 effectors; thus our studies with Foxp3<sup>+</sup> Tregs provide a more definitive assessment of Treg phenotype and function in the absence of Bcl6.

Defects in Treg cell development were not observed with Bcl6 deficiency, as we noted normal numbers of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs and Helios<sup>-</sup> CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> iTregs in the periphery in Bcl6-deficient mice. This is consistent with other reports in the literature indicating a dispensable role for Bcl6 in Treg development (Chung *et al.*, 2011; Yu *et al.*, 2009). *In vitro* assessment of Treg functionality also indicated intact suppression of T cell proliferative responses by Bcl6-deficient Tregs. In fact, Bcl6-deficient Tregs displayed augmented ability to suppress increased proliferation of Bcl6-deficient T cells, thereby also ruling out resistance of the Bcl6-deficient T cells to Treg-mediated suppression. We further noted that the Bcl6-deficient Tregs were capable of controlling Th1-mediated colitogenic responses *in vivo* when tested in the T cell transfer model of colitis. This indicated that their potent suppressor function



noted *in vitro* also translated *in vivo* with their observed competency to control Th1 responses. However, our studies in the Th2-driven allergic airway disease model showed the opposite effect. Mice receiving Bcl6-deficient Tregs displayed reduced lung function and striking increases in cellular infiltration in the BAL and the lungs, with predominance of eosinophils. The inflammatory response in the lungs was strongly Th2-biased with augmented levels of Th2 cytokines in the mice receiving Bcl6-deficient Tregs, even higher than the levels in the control group. Thus, our studies in the Th2 disease model highlighted that Bcl6-deficient Tregs were not simply defective in control of inflammatory Th2 responses; they actually augmented Th2 responses *in vivo*. Similar failure to control systemic Th2 responses was also noted with mixed chimeras of Foxp3-deficient (scurfy) bone marrow with Bcl6-deficient bone marrow. Foxp3 expression limits effector Th1, Th2 and Th17 production in Treg cells, resulting in a fulminant mixed T cell-driven pathology in scurfy mice (Koch *et al.*, 2009; Liston *et al.*, 2008; Singh *et al.*, 2007). Considering that chimeras of Bcl6-deficient BM with scurfy BM led to increased Th2-type T cell responses again highlights the selective failure of Bcl6-deficient Tregs to control Th2 responses *in vivo*.

An important issue is how Bcl6-deficient Tregs selectively fail and exacerbate Th2 responses in the airway inflammation and the Foxp3-deficient scurfy chimera models. Key to these questions was the Th2-bias noted for Bcl6-deficient Tregs by gene expression profiling. Bcl6-deficient Foxp3<sup>+</sup> Tregs expressed increased Th2 cytokines (*Il4*, *Il5*, *Il13*), Th2 transcription factors (*Gata3*, *Maf*) and chemokine receptor (*Ccr8*), while Th1 and Th17-associated

genes were not significantly different compared to wild-type Treg counterparts. Importantly, this observed increase in Th2 genes was not associated with down-regulation of Foxp3 or other Treg genes. Bcl6-deficient Tregs retained either intact or increased expression of Treg suppressor molecules (particularly, *Il10* and *Gzmb*). Thus, unlike other reports in the literature, wherein Treg cells lose Foxp3 expression in inflammatory contexts and acquire effector cytokine-producing abilities (Kitoh *et al.*, 2009; Wan and Flavell, 2007; Wang *et al.*, 2010), Foxp3<sup>+</sup> Tregs in the Bcl6-deficient mice up-regulated Th2 genes, indicative of their acquisition of a hybrid Treg-Th2 phenotype. This also suggested that Bcl6 regulates Th2 gene expression in Tregs, via Foxp3-independent mechanisms.

One obvious explanation for the exacerbated Th2 responses promoted by Bcl6-deficient Tregs could be their down-regulation of Foxp3 expression in the inflamed Th2 environment in the Th2 model. However, since we note higher Foxp3 expression in lungs of mice receiving Bcl6-deficient Tregs than wild-type Tregs (Figure 16), this possibility seems unlikely. Further, we have noted that the Foxp3<sup>+</sup> Bcl6-deficient Tregs secrete Th2 cytokines. Thus, the more interesting possibility is that Bcl6-deficient Tregs promote Th2 inflammation by secreting Th2 cytokines that mimic Th2 effector activity and/or amplify further Th2 differentiation, and that *in vivo* this effect over-rides the suppressive functions. While we have observed that supernatants from Bcl6-deficient Tregs can promote Th2 differentiation of naïve CD4<sup>+</sup> T cells, the amount of IL-4 and IL-5 secreted by Bcl6-deficient Treg cells is much less than what is made by activated Th2 cells *in vitro* (D.V.S., A.M., A.L.D., data not shown) and is unlikely to be

higher than what is produced by activated Th2 cells *in vivo*. Another possibility is that Bcl6-deficient Tregs migrate more efficiently to the inflamed organ than other cell types, due to increased expression of several chemokine receptors (Figure 19). Once in the lungs, the increased Th2 cytokines made by the Bcl6-deficient Tregs are likely to upset the normal regulatory balance. For instance, IL-4 can desensitize effector T cells to the inhibitory effects of Tregs (Pillemer *et al.*, 2009). Bcl6-deficient Tregs may produce other factors besides IL-5 that promote eosinophil recruitment, however neither eotaxin-1 (Ccl11), eotaxin-2 (Ccl24) nor eotaxin-3 (Ccl26) mRNAs were significantly increased in the Bcl6-deficient Tregs (D.V.S., A.L.D., data not shown).

Nevertheless, these experiments assessing the phenotype and function of Bcl6-deficient Tregs highlighted the critical role for Bcl6 in repressing Th2 gene expression in the Treg lineage, similar to conventional T cells. Thus, in the absence of Bcl6, Tregs develop a Th2-type pro-inflammatory phenotype, showing that Bcl6 is a key transcriptional regulator of Treg function. The selective failure to control Th2 responses *in vivo* can explain the hallmark Th2-mediated heart and lung pathology that develops spontaneously in Bcl6-deficient mice. Indeed, our attempts to characterize Tregs isolated from the heart and lungs of Bcl6-deficient mice revealed their potent Th2-bias associated with secretion of Th2 cytokines and an increase in the numbers of IL-4<sup>+</sup>Foxp3<sup>+</sup> Tregs relative to wild-type counterparts (data not shown). Thus, these hybrid Th2-Tregs may offset the regulatory balance in these organs and contribute to the exacerbations to the disease phenotype noted in the absence of Bcl6. Thus, expression of Bcl6

in Tregs is essential for Treg-mediated control of Th2 inflammatory responses and to limit excessive pro-Th2 activity of this regulatory subset *in vivo*.

### **Bcl6 limits the Th2 inflammatory activity of Tregs by repressing the transcriptional activity of Gata3**

While Foxp3 is central to the Treg phenotype and function, Foxp3 alone does not explain the complete transcriptional landscape of this crucial regulatory subset (Chatila, 2007; Hill *et al.*, 2007). Several other transcription factors have emerged that regulate Treg cell development, homeostasis and suppressor function (Campbell, 2011; Rudensky, 2011). The variegated expression of transcription factors, chemokine receptors, and suppressor modules allow for phenotypic and functional specialization of Treg cells that enable optimal control of diverse immune challenges. The current dogma in the Treg field indicates that Treg cells undergo functional specialization in the periphery by co-opting the transcriptional program of the effector T cells they suppress. Thus, many transcription factors have been identified that control the ability of Tregs to suppress a specific type of immune response (T-bet for Th1 responses, Irf4 for Th2 responses, Stat3 for Th17 responses and Bcl6 for Tfh and GC B cell responses) (Chaudhry *et al.*, 2009; Chung *et al.*, 2011; Koch *et al.*, 2009; Linterman *et al.*, 2011; Zheng *et al.*, 2009). Most relevant to our observations is the requirement of Irf4 in Tregs for effective control of Th2 responses. However, the expression of *Irf4* was unaltered in the absence of Bcl6 in Tregs. The pathology in Irf4-deficient mice was localized to pancreas, lungs and stomach, unlike the heart and lung-specific disease noted in Bcl6-deficient mice. Also, while Irf4-deficient Tregs fail to suppress autoimmune Th2 responses, Irf4-deficient Tregs do not express elevated Th2 genes (Gata3 or Th2 cytokines), as

noted for Bcl6-deficient Tregs. Irf4-deficient Tregs however, display reduced expression of Treg suppressor genes (particularly *Il10* and *Gzmb*) and also reduced chemokine receptor, *Ccr8* expression. Thus, the reduced expression of Treg suppressor genes has been proposed to contribute to the observed Th2 pathology in Irf4-deficient mice. Thus, these differences in the observed pathology and the Treg phenotype in the absence of Bcl6 (increased Th2 genes, increased Treg suppressor genes like *Il10* and *Gzmb* as well as increased *Ccr8* expression) are consistent with Bcl6 and Irf4 operating through distinct mechanisms in Tregs.

Tregs cells have previously been reported to exhibit an intrinsic Th2-bias, associated with higher expression of the Th2 transcription factor, Gata3 in Tregs, compared to conventional T cells (Wang *et al.*, 2010). The inherent Th2 bias is partially attributed to the ability of Foxp3 to up-regulate Gata3, but not the Th1 transcription factor, T-bet. Two recent reports reported a functional role for the Treg-specific Gata3 expression using Treg-specific Gata3 mutant mice (Wang *et al.*, 2011; Wohlfert *et al.*, 2011). Both mouse and human Tregs express Gata3, particularly at mucosal surfaces. Wang *et al* noted that mice with Gata3-null Tregs develop spontaneous inflammation at multiple sites, even under homeostatic conditions, while Wohlfert *et al* indicated that Gata3 played a critical role in Treg physiology during inflammation. Nevertheless, both these studies demonstrated that Gata3-deficient Tregs have reduced expression of *Foxp3* and Treg suppressor genes (*Ctla4*, *Gitr*, *Il2ra*) and expressed Th1, Th2 and Th17 effector cytokines. Thus, unlike T-bet or Stat3 expression in Tregs, Gata3 seems

to play a broader role in terms of Treg-mediated control of Th1, Th2 and Th17 immune responses. Treg-specific expression of Gata3 is induced by TCR stimulation and occurs independent of IL-4/STAT6 signaling, unlike conventional T cells. The key question that these studies pose is identifying the signals that regulate the expression of Gata3 in Tregs and prevents the expression of Th2 cytokines by Gata3 in Tregs.

Bcl6 is well established as a potent inhibitor of Th2 differentiation and Th2-mediated inflammation. This regulation occurs via an IL-4/STAT6 independent mechanism and involves post-transcriptional repression of the Th2 transcription factor, Gata3 by Bcl6 (Kusam *et al.*, 2003). Bcl6 over-expression has been demonstrated to inhibit non-Tfh fate determination by repressing the expression of *Tbx21*, *Gata3* and *Rorc* or by blocking the transcriptional activity of T-bet and RORyt (Nurieva *et al.*, 2009; Yu *et al.*, 2009). However, while Bcl6 represses Gata3 protein, its ability to block transcriptional function of Gata3 has not been ascertained in previous reports. We observed that Bcl6 potently represses the Th2 cytokine-inducing ability of Gata3 in two independent assays. Similar results were also noted in B cells, indicative that Bcl6 represses Gata3 function independent of other T cell factors. Thus, these studies confirm that Bcl6 limits the transactivation of Th2 cytokines by Gata3 in T cells. Gata3 can auto-activate its own expression (Ouyang *et al.*, 2000). Thus, the increased Gata3 expression noted in Bcl6-deficient Tregs may be explained by the augmented ability of Gata3 to auto-activate its own expression in the absence of Bcl6, but also probably reflects the Th2 environment and the degree of Treg stimulation through

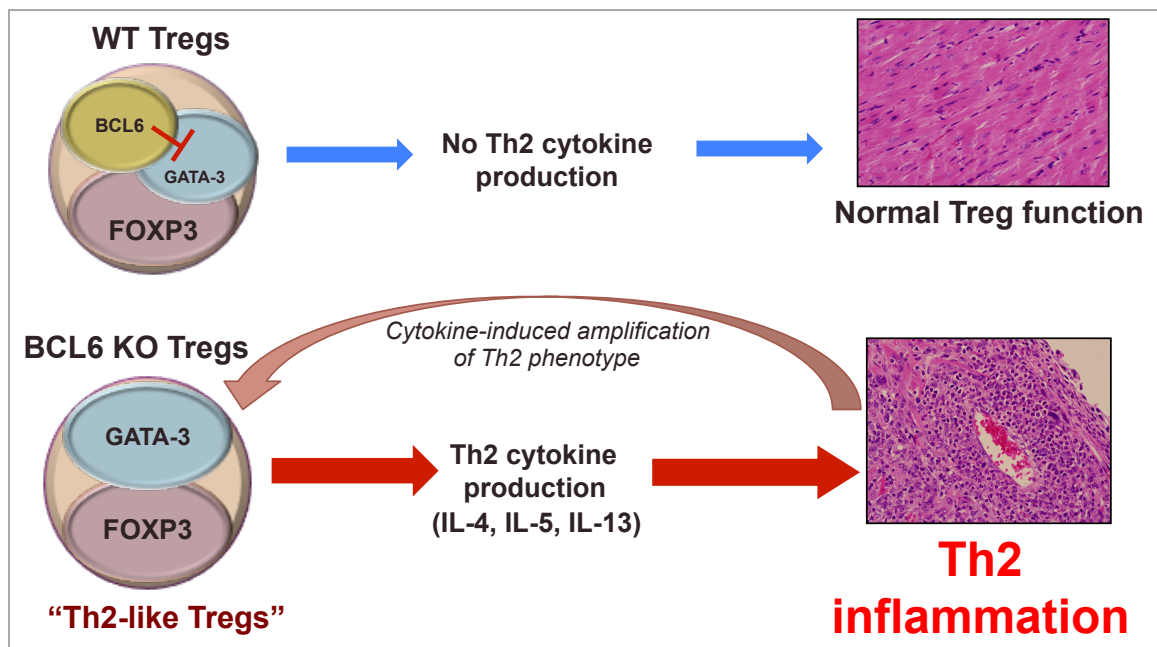
the TCR. From our current findings, we propose that Bcl6 controls Gata3 expression in Tregs independently of IL-4 and Stat6, by dampening Gata3 transcriptional activity and modulating the ability of Gata3 to auto-activate its own expression.

Thus, our studies with Bcl6-deficient Tregs suggest that Bcl6 is one of the key factors regulating Gata3 function in Tregs. This limits the transactivation of Th2 cytokines by Gata3 in Tregs, thereby preventing the Tregs from converting to pro-inflammatory Th2-type Tregs, exacerbating Th2 pathologies *in vivo* (Figure 54). Acquisition of specific T effector transcriptional programs allows Tregs to effectively curb immune responses, however this regulatory mechanism can lead to pathogenic consequences, due to the presence of self-reactive TCRs on Tregs. Thus, Treg-acquired effector features need to be under tight control, and here we have shown that a Bcl6-mediated brake on Gata3 activity in Tregs is essential for the ability of Tregs to control Th2-type inflammation. Bcl6 is unique among transcription factors that regulate the ability of Tregs to control Th2 responses; in that Bcl6 represses Gata3 and Th2 responses in Tregs at the same time that Bcl6 is required for Tregs to properly control Th2 cell responses. Bcl6 thus prevents Tregs from acquiring Th2 effector-like characteristics, by repressing Gata3 function.

Our study provides an interesting new facet to the Treg-transcription factor driven suppression model wherein expression of T helper specific transcription repressors such as Bcl6 is essential for Tregs to control inflammatory T cell responses that are inhibited by the same repressor. Whether the control of Th2



responses by Bcl6 in Tregs is unique or similar repressor factors exist for control of Th1 and Th17 responses by Tregs will definitely spark interest in the Treg field and call for further investigations. Also one important point that derives from this work is that Bcl6 control in Tregs regulates the rare autoimmune disease, myocarditis. Thus investigating the significance of other such transcriptional repressors has the potential to identify an entirely new realm of rare autoimmune diseases subject to Treg control mechanisms.



**Figure 54: Repression of Gata3 function by Bcl6 limits Th2 gene expression in Tregs.**

Bcl6 expression in the Treg lineage limits the transcriptional activity of Gata3, thus controlling expression of Th2 cytokines in Tregs. Thus, wild-type Tregs retain normal Treg suppressor function. In the absence of Bcl6 in Tregs, Tregs acquire a Th2-bias due to increased Gata3 activity, produce Th2 cytokines and augment Th2 responses *in vivo*. This leads to exacerbated Th2 inflammatory responses, that manifest as myocarditis in the absence of Bcl6.

## **Bcl6 limits the Th2 inflammatory activity of Treg cells by repressing miR-21**

MicroRNAs have emerged as critical regulators of Treg lineage stability, particularly under inflammatory contexts (Liston *et al.*, 2008; Zhou *et al.*, 2008b). The lack of any single Treg suppressor mechanism does not recapitulate the fatal autoimmunity that develops spontaneously in Foxp3-deficient scurfy mice. However, deletion of miRNA-processing enzymes in the Treg lineage completely phenocopies the disease in scurfy mice, thus highlighting the importance of miRNAs in Treg suppressor function. A number of miRNAs are differentially expressed in Tregs compared to conventional T cells in a Foxp3-dependent manner (Cobb *et al.*, 2006). Recent studies are defining the contribution of individual miRNAs to the Treg phenotype and function that will enable fine-tuning Treg activity in diverse disease settings.

Relevant to our study was the observation that miR-146a was preferentially expressed in Tregs and regulated Treg-mediated control of Th1 inflammatory responses. Mice harboring miR-146a-deficient Tregs displayed increased activation of the target gene (Stat1) (Lu *et al.*, 2010). Thus, the miR-146a-deficient Tregs were Th1-biased, secreted the Th1 cytokine, IFN $\gamma$  and failed to suppress Th1 immune responses. Our results with Bcl6-deficient Tregs displayed a similar paradigm for the control of Th2 responses. Bcl6-deficient Tregs were Th2 biased, secreted Th2 cytokines and failed to suppress Th2 responses. In addition, Bcl6 was demonstrated to repress miRNAs in T cells to allow Tfh fate determination (Yu *et al.*, 2009). These results suggested that Bcl6 controls Treg stability by repressing miRNAs in Treg cells. Indeed, miRNA

profiling identified a signature of miRNAs that are differentially expressed in Bcl6-deficient Tregs. Our studies suggest that miR-21 was highly expressed in Bcl6-deficient Tregs. MiR-21 is a Stat3-regulated miRNA and thus pro-inflammatory cytokines such as IL-6 up-regulate miR-21 via Stat3 during inflammation (Iliopoulos *et al.*, 2010; Loffler *et al.*, 2007; van der Fits *et al.*, 2011). Here we have found that Bcl6 represents a counter-balance to up-regulation of miR-21 by Stat3, particularly in regulatory T cells, which are necessary for controlling inflammation. Intriguingly, Stat3 and Bcl6 appear to bind to a common site in the miR-21 promoter, indicating the potential for competitive binding of these factors to miR-21 in cells in an inflammatory environment.

In addition to identifying the Bcl6-Stat3 pathway that can regulate miR-21 expression in Tregs during inflammatory settings, we also extend our study to identify a novel role for miR-21 in T cell differentiation. Previous reports have suggested that miR-21 expression in myeloid cells inhibits the Th1 cytokine, IL-12 and indirectly promotes Th2 responses (Lu *et al.*, 2011b; Lu *et al.*, 2009b). MiR-21 positively regulates the Ras-MAPK pathway by targeting negative regulators of the Ras-MAPK pathway such as *Spry1*, *Spry2*, *Btg2* and *Pdcd4* (Jung and Calin, 2010). Of these target genes, *Spry1* mRNA was significantly decreased in Bcl6-deficient Tregs. Thus, we identify a T cell-intrinsic role for miR-21 in promoting Th2 differentiation, likely by targeting the Map kinase pathway inhibitor (*Spry1*). Since the master Th2 factor Gata3 can be stabilized by Map kinase activity (Yamashita *et al.*, 2005), our studies suggest that the increased miR-21 can decrease *Spry1*, thus promoting greater Map kinase activity and thus

increased Gata3 and Th2 differentiation. This miR-21-MAP kinase mechanism could account for some of the ability of Bcl6 to repress Gata3 activity in the retroviral and reporter assays.

Our results imply that T cells within an inflammatory environment can undergo Th2 differentiation due to miR-21 up-regulation, and that this is enhanced in the absence of Bcl6. The deregulation of miR-21 in Bcl6-deficient T cells could augment Th2 differentiation, and lead to deregulated Th2 inflammation as is seen in Bcl6-deficient mice. Thus, miR-21 may be part of a positive feedback loop for Th2 inflammation, which can explain the large increase in miR-21 in wild-type Tregs in the Bcl6-deficient environment. The deregulation of miR-21 in Bcl6-deficient Tregs could accelerate the positive feedback of Th2 inflammation and lead to greater miR-21 induction within Bcl6-deficient Tregs, leading to higher levels of Th2 gene expression. Since miR-21 regulates Th2 differentiation by a separate pathway than the canonical pathway of IL-4 and Stat6, our findings may explain why Th2-type inflammation occurs in Bcl6-deficient mice independently of IL-4 and Stat6 (Dent *et al.*, 1998a). Repression of miR-21 by Bcl6 might also explain the post-transcriptional control of Gata3 and Th2 differentiation by Bcl6 (Kusam *et al.*, 2003). Stat3 is required for full Th2 differentiation (Stritesky *et al.*, 2011), and the failure to up-regulate miR-21 can explain the incomplete Th2 differentiation seen with Stat3-deficient T cells. The counter-regulation of miR-21 by Bcl6 and Stat3 can explain the opposing roles of these transcription factors in Th2 differentiation, with Bcl6 inhibiting and Stat3 promoting Th2 differentiation, respectively.

Interestingly, the regulation of miR-21 by Bcl6 may also relate to the specific development of myocarditis in Bcl6-deficient mice, as increased miR-21 expression in cardiac fibroblasts has been associated with cardiac hypertrophy. MiR-21 has been shown to regulate heart diseases by regulating the ERK-MAP kinase pathway in cardiac fibroblasts, impacting global cardiac structure and function (Thum *et al.*, 2008). MiR21 levels are selectively increased in fibroblasts of failing hearts, leading to inhibition of a miR21 target gene, *Spry1*, that negatively regulates the ERK-MAP kinase-signaling cascade. Thus, a de-regulated miR-21-*Spry1* pathway in Bcl6-deficient Tregs could also contribute to the exacerbations of myocarditis associated with the absence of Bcl6 (Figure 55).

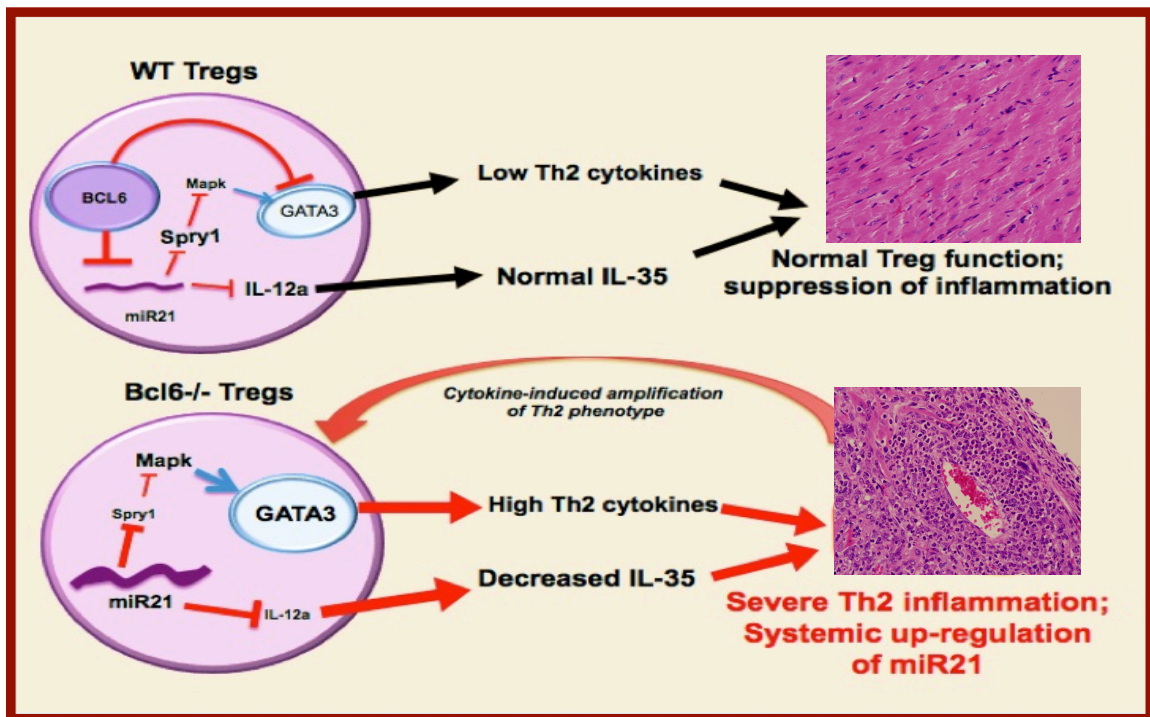
MiR-21 is specifically increased in Tregs versus conventional T cells (Cobb *et al.*, 2006), suggesting that miR-21 may have an important role in Treg biology. MiR-21 is up-regulated in human Tregs, and can positively regulate Foxp3 expression (Rouas *et al.*, 2009). Since many Treg suppressor genes are dependent upon Foxp3 for their expression, regulation of miR-21 by Bcl6 can control expression of Foxp3 and thus other Treg genes. Indeed, the increased miR-21 can account for elevated Treg suppressor genes (*Foxp3*, *Il10*, *Ctla4*, *Tgfβ*) noted in Bcl6-deficient Tregs. IL-10 is a well-characterized Bcl6 target gene in T cells (Kusam *et al.*, 2003) and is positively regulated by miR-21 (Sheedy *et al.*, 2010). Thus Bcl6 may regulate IL-10 indirectly in Tregs via miR-21 regulation. Interestingly, our results also demonstrate that miR-21 can also decrease *Il12α* expression in Treg cells. *Il12α* (p35 subunit of IL-12) is a component of the Treg immune-suppressive cytokine, IL-35, in conjunction with *Ebi3* (Collison *et al.*,

2007). Thus, reduced *Il12a* expression in Bcl6-deficient Tregs may lead to less IL-35 secretion. IL-35 is important for control of allergic diseases in a house-dust mite allergen challenge model (Huang *et al.*, 2011). Thus, the reduced IL-35 secretion from Bcl6-deficient Tregs may further contribute to their failure to control Th2 responses (Figure 55). Overall, the constellation of gene changes mediated by increased expression of miR-21 can potentially explain the unusual gene signature exhibited Bcl6-deficient Tregs with augmented Treg and Th2 genes resulting in potent suppression of T cell proliferation *in vitro* yet exacerbated Th2 inflammation *in vivo*.

MiR-21 is increased in a large number of different pathologic conditions, typically on the order of 2- to 6-fold (Liu *et al.*, 2010; Pan *et al.*, 2010; Si *et al.*, 2007; Stagakis *et al.*, 2011; Thum *et al.*, 2008; van der Fits *et al.*, 2011; Wu *et al.*, 2008). In previous studies on Th2-type airway inflammation, miR-21 was up-regulated 3- to 6-fold (Lu *et al.*, 2012a; Lu *et al.*, 2009b; Lu *et al.*, 2012b). In our work, we observed a 9-fold increase in miR-21 in the severe Th2-type airway inflammation induced with Bcl6-deficient Treg cells. In ulcerative colitis, a Th2-type inflammatory disease of the colon, miR-21 was elevated up to 20-fold in actively inflamed disease tissue (Wu *et al.*, 2008). While two other studies report an increase in miR-21 in biopsies of patients with EoE, the fold induction reported was about 4-fold (Lu *et al.*, 2012a; Lu *et al.*, 2012b), while miR-21 was reported as undetectable in the patient sera (Lu *et al.*, 2012b). The 50-fold average increase in miR-21 in EoE biopsies and 30-fold average increase in circulating miR-21 in EoE in our study represents one of the greatest elevations of miR-21 in

diseases reported in the literature. We reason that differences in the type of control individuals selected in our study may account for the discrepancy in the two studies. Since the controls used in the Lu *et al* study were enriched for atopy (Lu *et al.*, 2012b), unlike our study, we tested miR-21 levels in the sera of asthmatic individuals and noted a 4-fold induction of miR-21. We thus propose that high levels of miR-21 are a novel biomarker for severe Th2-inflammation, and that highly elevated serum miR-21 may be useful for diagnosis of Th2-inflammatory disease. While miR-21 is a well-known “oncomir” due to its over-expression in multiple types of cancer (Hatley *et al.*, 2010), miR-21 is also commonly de-regulated in inflammatory diseases, and thus could also be termed as an “inflamir”.

While miR-21 function has been extensively studied in cancer, the function of miR-21 in T cells and inflammation has not been well characterized. To our knowledge, this is the first study identifying miR-21 as a novel target gene for Bcl6 in Treg cells, and reporting a T cell autonomous role for miR-21 in promoting Th2 differentiation. Finally, we have identified miR-21 target genes that shed light on the role of miR-21 in conventional T cells and Tregs. In addition to defining a functional role for miR-21 in Th2 inflammatory conditions, we also extend our study to characterize miR-21 as a non-invasive biomarker for human Th2 diseases (like EoE and asthma). Thus, repression of miR-21 by Bcl6 in Tregs adds another important facet to the Treg-mediated control of inflammatory Th2 responses.



**Figure 55: Repression of miR-21 by Bcl6 limits Th2 gene expression in Tregs.**

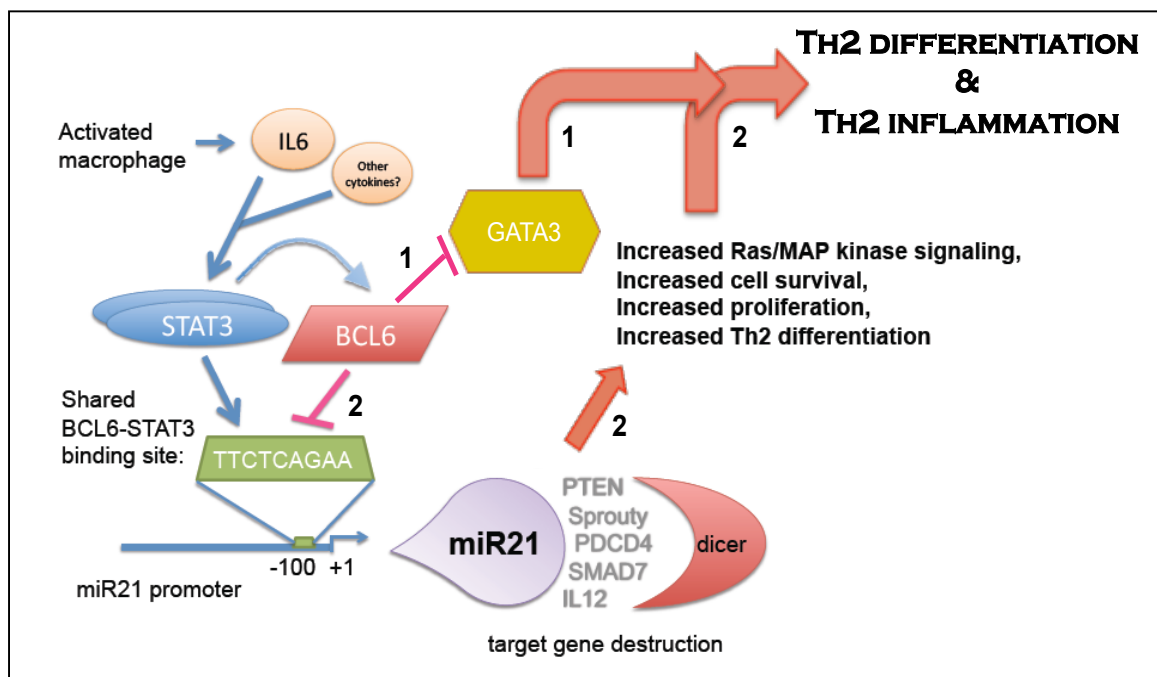
Bcl6 expression of miR-21 in the Treg lineage limits Gata3 expression via *Spry1*-Mapk pathway, thus controlling expression of Th2 cytokines in Tregs. *Il12a* levels are also maintained, allowing for optimal secretion of IL-35, that permits effective control of Th2 inflammation. In the absence of Bcl6 in Tregs, the increased miR-21 promotes increased Gata3 and Th2 gene expression, promoting a Th2-bias in the Tregs and failure to control Th2 responses *in vivo*. Also reduced *Il12a* levels with increased miR-21 lead to less IL-35 secretion, that further exacerbates Th2 inflammatory responses *in vivo*.



## **Intrinsic and extrinsic regulation of Th2 gene expression by Bcl6 in Tregs and their contributions to the Th2 inflammatory disease in Bcl6-deficient mice**

Bcl6 is expressed in multiple cell types to different extents (including B, T and myeloid cells), although its expression is particularly high in GC B and Tfh cells. Thus, the inflammatory disease in germ-line Bcl6-deficient mice is complex in nature and involves contributions from diverse cell lineages (Toney *et al.*, 2000; Yoshida *et al.*, 1999). The disease is primarily T cell-dependent as Bcl6/Tcra double-deficient mice fail to develop spontaneous myocarditis and pulmonary vasculitis. The ability of Bcl6 to inhibit Th2 differentiation by repressing the Th2 factor, Gata3, can explain the strong Th2-bias of the inflammatory disease noted in Bcl6-deficient mice (Kusam *et al.*, 2003). In addition, functions of Bcl6 in the myeloid lineage also contribute to the excessive eosinophilic infiltration and hyper-IgE phenotype (Toney *et al.*, 2000; Yu *et al.*, 2005). Deregulated chemokine expression by macrophages in Bcl6-deficient mice also promotes the Th2 inflammation (particularly since MCP-1, MRP-1, MCP-3 favor Th2 polarization via effects on APCs) (Toney *et al.*, 2000). Bcl6 also seems important to protect mature cardiac myocytes from degeneration (Yoshida *et al.*, 1999). Since the disease develops spontaneously, is T cell-dependent and resembles the inflammation noted in mice with defects in Treg suppressor molecules like CTLA-4 or TGF $\beta$  (Kulkarni and Karlsson, 1993; Tivol *et al.*, 1995), our study investigated the contributions of defects in the Treg lineage to the disease in Bcl6-deficient mice. Our results extend the role of Bcl6 in inhibiting

Th2 differentiation in T cells to Treg cells. Thus, we demonstrate a critical role for Bcl6 in repressing Th2 gene expression in Treg cells and preventing their conversion to pro-inflammatory Th2 effectors. We have identified two different mechanisms that can explain the Th2-bias exhibited by Bcl6-deficient Tregs and their selective failure to control Th2 inflammatory responses: (1) Increased Gata3 transcriptional activity in the absence of Bcl6 can account for the increased Th2 cytokine expression and also increased Gata3 expression noted in Bcl6-deficient Tregs, (2) Increased miR-21 expression in the absence of Bcl6 can explain the Th2-bias, since miR-21 can polarize T cells to Th2 fate, potentially via our proposed *Spry1*-Mapk-Gata3 pathway (Figure 56).



**Figure 56: Control of Th2 gene expression and Th2 inflammatory activity of Treg cells by Bcl6.**

Bcl6 functions in Treg cells by 2 different mechanisms to limit their acquisition of a pro-Th2 inflammatory phenotype: (1) Inhibits Gata3 function, thus preventing secretion of Th2 cytokines by Tregs, and (2) Competes with Stat3 for repression of miR-21. MiR-21 can target *Spry1* and *I112a*, and thus promote Th2 differentiation of naïve T cells. Inflammatory cues (IL-6 acting via Stat3) up-regulate miR-21 and this combined with Bcl6 deficiency exacerbates Th2 differentiation and Th2 inflammation.

Treg cells get remodeled in inflammatory environments; they lose Foxp3 expression and/or acquire effector T cell features (Zhou *et al.*, 2009b). Thus, considering the hyper-Th2 inflammatory milieu in Bcl6-deficient mice, it is essential to delineate the intrinsic effects due to absence of Bcl6 in Treg cells and assess indirect contributions from extrinsic inflammatory signals. One approach we took in this regard included evaluating Bcl6-deficient Treg phenotype from mixed chimeras (WT BM:KO BM) (Figure 21). Increased expression of Gata3, IL-4 and miR-21 in Bcl6-deficient Tregs derived from the chimeras relative to their wild-type Treg counterparts validates the intrinsic role of Bcl6 in repressing Th2 genes and miR-21 in Treg cells (Figures 22 and 27). However, we observed that the overall expression of Th2 genes in chimera-derived Bcl6-deficient Tregs was significantly reduced compared to Tregs from germ-line Bcl6-deficient mice (Figure 19). This can be explained by the presence of functional wild-type Tregs in the mixed WT:KO chimeras that dampen the overall severity of Th2 inflammation. Indeed, the Bcl6-deficient Tregs derived from KO:Foxp3KO chimeras were strongly Th2-polarized similar to those in germ-line KOs, as compared to Tregs from WT:Foxp3KO chimeras (Figure 23). The Th2-bias noted in the Stage I conditional knockout Tregs and increased percentages of GATA-3<sup>+</sup>Foxp3<sup>+</sup> Tregs in the allergic airway inflammation model with the final Stage III conditional mutant mice provides a definitive validation to the intrinsic role of Bcl6 in limiting Th2 gene expression and Treg control of Th2 responses (Figures 49 and 51). Overall, the Th2-bias noted for the conditional knockout Tregs was reduced compared to germ-line Bcl6-deficient mice. Taken

together the bone marrow chimera and the conditional Bcl6-knockout Treg data validate the intrinsic role of Bcl6 in the Treg lineage in repressing Th2 genes and miR-21.

At the same time, relatively reduced Th2 polarization in both the chimera-derived and the conditional Bcl6-deficient Tregs suggests that the Th2 inflammatory disease in Bcl6-deficient mice also contributes to the remodeling of the Treg phenotype. Indeed, the observation that the stable Tregs and the exTregs in Bcl6-deficient mice (KO RFC) acquire a potent Th2-bias does highlight the effect of the Th2 inflammatory environment on Bcl6-deficient Treg phenotype (Figure 46). In addition, we noted increased exTregs (loss of Foxp3) and reduced stable Tregs in KO RFC mice, relative to WT RFC (Figure 45). Presence of the NOD background in the BRFC mice can account for their more severe Th2 disease compared to germ-line Bcl6-deficient mice, and can accelerate exTreg generation in the absence of Bcl6. However, the striking Th2-bias noted in the exTregs and even the stable Tregs does validate the influence of the environment on Bcl6-deficient Treg phenotype and subsequent suppressor function. Also, we note acquisition of miR-21 expression by wild-type Tregs transferred to Bcl6-deficient mice, relative to wild-type mice (Figure 32). Thus, in inflammatory settings, Tregs can acquire miR-21 expression and its expression is further up-regulated in Tregs in the absence of Bcl6. Thus, overall we have demonstrated that the Bcl6-deficient Treg phenotype is a composite effect of loss of intrinsic regulation by Bcl6 and extrinsic effects from the inflammatory Th2 disease. Both of these effects amplify the Th2 gene and miR21 expression and

thereby contributes to the failure of Bcl6-deficient Tregs to control Th2 responses.

The observation that the hypomorphic stage I Treg-specific conditional mutant mice also develop heart inflammation similar to the germ-line Bcl6-deficient mice validates defective control of Th2 responses by Bcl6-deficient Tregs. However, since the final Stage III Treg-specific conditional mutant mice do not develop heart pathology indicates that the pro-Th2 Bcl6-deficient Tregs do not initiate the heart inflammation, but fail to suppress aberrant Th2 responses that develop from absence of Bcl6 in diverse cell lineages. Thus we propose that the absence of Bcl6 in cardiac myocytes initiates degenerative changes in the cardiac myocardium. These changes trigger infiltration of inflammatory Th2 cells and eosinophils. The Th2 cytokines and the toxic eosinophil granule components exacerbate the heart inflammation. Bcl6-deficient Tregs get recruited to the inflamed hearts, but these Th2-biased Tregs undergo further remodeling in the inflammatory Th2 milieu and their expression of Th2 genes and miR-21 offsets the regulatory balance and contributes to their failure to suppress myocarditis. Also the fact that the conditional knockout mice do not develop lung inflammation as the germ-line Bcl6-deficient mice, indicates that loss of Bcl6 in the other lineages (particularly the myeloid lineage) is likely a key factor for the lung vasculitis. Thus assessing the phenotype of the myeloid, T and B cell-specific conditional Bcl6 knockout mice strains will provide further insights into the contributions of individual cell types to the myocarditis and pulmonary vasculitis in germ-line Bcl6-deficient mice.

## **FUTURE DIRECTIONS**

### **Analyze the role of miR-21 in Th2-type inflammation**

We will use gain-of-function and loss-of-function approaches to perform an in-depth assessment of the role of miR-21 in Th2-type inflammation. Our studies so far have demonstrated that miR-21 can prime *de novo* differentiation of naïve T cells to Th2 fate and that increased levels of miR-21 are observed in Th2 pathologies in both mice and human. One of the key questions to be addressed includes determining whether increased miR-21 is a passive marker associated with Th2 diseases or actively responsible for the initiation and exacerbations of the Th2 pathologies. Thus, to better understand the role of miR-21, we will analyze the signals that control miR-21 expression, cell types that express miR-21 in Th2 inflammation, and miR21 target genes that can provide mechanistic insights into its function. Finally, we should analyze miR-21 in a Th1 model of inflammation to test whether miR-21 is increased to a greater extent in Th2 inflammation than other inflammatory conditions. We also want to delineate the contributions of increased miR-21 to Bcl6-deficient Treg phenotype and their failure to suppress Th2 responses *in vivo*. We have already obtained miR-21 transgenic (miR-21 Tg), miR-21 knock-out (miR-21 KO) and miR-21 floxed and conditional miR21 Tg mice from Dr. Eric Olson at UT Southwestern, and these mouse models will be ideal tools to gain definitive insights into the function of miR-21 in Th2 inflammatory settings *in vivo*.

## **Assess effects of miR21 over-expression and knock-down in mouse model of allergic airway inflammation**

Here we will use cohorts of wild-type and miR-21 Tg mice and assess induction of Th2 inflammation in the Ova-induced airway inflammation model (Figure 12). Additionally, unchallenged mice of each type will be assessed to see whether basal lung inflammation is altered in the miR-21 Tg mice. Parameters to be analyzed are BAL cell counts, BAL cell analysis by flow cytometry, cytokine levels in lung and draining lymph node by QPCR (*Il4*, *Il5*, *Il13*, *Il17*, *Ifng*, *Tnfa*, *Ccl2*, *Ccl3*, *Ccl11*), lung histology, serum IgE levels, and enhanced pause (Penh) measurements following methacholine challenge by whole body plethysmography. We predict that overexpression of miR-21 would promote augmented Th2 allergic responses with increased inflammation and airway hyper-reactivity. A similar set of experiments will also be performed using cohorts of wild-type and miR-21 KO mice, which would develop reduced Th2 responses compared to controls, thus confirming that miR-21 is functionally implicated in Th2 pathologies *in vivo*.

### **Assess cell types that express miR-21 in allergic inflammatory settings**

MiR-21 expression in myeloid cells was reported to inhibit the Th1 cytokine, IL-12 and indirectly prime for Th2 responses (Lu *et al.*, 2011b; Lu *et al.*, 2009b). However, our studies have identified a T-cell intrinsic role for miR-21 in promoting Th2 differentiation. In addition, our studies show significant miR-21 expression in the lung epithelial cells. Hence, understanding the cell-types that contribute to its effects in allergic settings would be important for developing strategies to inhibit miR-21. The conditional miR-21 Tg and KO mice, that allow for over-expression and deletion of miR-21 in specific cell-types by mating to tissue-specific cre mice, would provide useful insights into the functional relevance of miR-21 in different cell types. We can therefore generate CD4 T cell, Treg, myeloid and epithelial cell type-specific miR-21 Tg and KO mice by crossing to CD4-cre, Foxp3-cre, lysM-cre and surfactant protein C (SP-C) Cre, respectively. These mice would be tested for induction of allergic airway inflammation. Alternatively, BM chimeras could be produced with miR-21 Tg and KO BM into wild-type mice, to assess the effect of miR-21 in hematopoietic cells versus lung stromal cells. These experiments will provide clear understanding of the cell-type specific role of miR-21 that can aid in developing strategies targeting this miRNA to attenuate allergic responses *in vivo*.



### **Assess relevance of miR-21 to the exacerbations of allergic inflammatory responses promoted by Bcl6-deficient Tregs *in vivo***

Our studies with the airway inflammation model demonstrated that transfer of Bcl6-deficient Treg cells promoted exacerbated Th2 responses that were associated with almost 10-fold induction of miR-21 in both the lungs and the sera of the recipient mice. Therefore, to define the contribution of miR-21 in the increased Th2 inflammatory responses promoted by Bcl6-deficient Tregs, we will generate Bcl6/miR-21 double KO (dKO) mice. These dKO Tregs will be tested in the airway inflammation model (as shown in Figure 12). In addition, we will transfer Bcl6-deficient Tregs into whole body miR-21 KO mice, and test induction of lung inflammation alongside wild-type mice that receive Bcl6-deficient Tregs. These experiments will determine if expression of miR-21 in non-Treg cells contributes to the disease exacerbations promoted by Bcl6-deficient Tregs.

### **Assess effect of miR-21 inhibitors in allergic inflammatory diseases**

To assess if inhibition of miR-21 has therapeutic implications in the treatment of allergic diseases, we will administer miR-21 inhibitors during the airway inflammation experiments. Locked nucleic acid (LNA)-based cholesterol tagged antisense miR-21 oligos that are effective at sequestering miR-21 by forming duplexes with miR-21 will be used for miR-21 inhibition *in vivo* (Liu *et al.*, 2010). A scrambled non-specific oligo will be used as a control for these experiments. These oligos will be instilled either during the initial OVA priming or the aerosol challenges by administration intra-tracheally that ensure delivery to

the lungs. Assessment of airway induction and Th2 inflammatory responses in mice receiving the anti-sense miR-21 and control probes will advance development of specific targeting strategies for miR-21 to treat allergic inflammation.

### **Assess miR-21 gene targets that offer mechanistic insights into its role in promoting Th2 responses**

MiR-21 has been demonstrated to target the myeloid cell-derived Th1 cytokine component, *Il12a*, in a previous report (Lu *et al.*, 2011b; Lu *et al.*, 2009b). Our studies in the airway inflammation model with Bcl6-deficient Tregs associated with increased miR-21 have validated *Il12a* as a target gene for miR-21 in myeloid cells. In addition, we have also identified the Mapk pathway inhibitor, *Spry1*, as miR-21 gene target, in the context of its role in promoting Th2 differentiation intrinsically in T cells. Lungs from miR-21 Tg mice tested for airway inflammation will be useful to validate miR-21 target genes. In addition, similar validation of cell-type specific miR-21 gene targets can be performed on lung tissue isolated from CD4, Foxp3, LysM and SpC-miR21 Tg mice following induction of airway inflammation. Biochemical analysis of our proposed miR-21-*Spry1*-Mapk-Gata3 pathway could be performed using approaches to overexpress miR-21 and *Spry1* and assess activation of Map kinases (phospho ERK1/2) and Gata3 protein levels. We expect that miR-21 overexpression in T cells would lead to reduced *Spry1* protein and increased phosphoERK and thus increased Gata3 and Th2 differentiation. On the other hand, overexpression of

*Spry1* will induce the opposite trend – reduced phosphoERK and thus decreased Gata3 and Th2 genes. These experiments using miR-21 Tg mouse strains and dissection of the miR-21-*Spry1* signaling pathway will provide mechanistic insights into its function, that can help in the development of therapeutic strategies.

**Assess the effect of Treg-specific miR-21 expression on the immune suppressive cytokine, IL-35 and its role in control of Th2 responses**

We have noted reduced *Il12a* expression in Bcl6-deficient Tregs that exhibit increased levels of miR-21. Hence we proposed that *Il12a* is a miR-21 gene target in Treg cells. Thus, increased miR-21 would lead to decreased levels of the Treg suppressive cytokine, IL-35 (composed of *Ebi3* and *Il12a* subunits) (Collison *et al.*, 2007). This observation can be validated by testing Treg supernatants from miR-21 Tg and KO mice, particularly, the Foxp3 Cre miR21 Tg and KO. These studies will identify a novel regulator for the Treg cytokine, IL-35. In addition, IL-35 has been implicated in the control of Th2 responses in the house dust-mite allergen challenge model *in vivo* (Huang *et al.*, 2011). Thus, testing the levels of IL-35 in our airway inflammation model with miR21 Tg and KO mice will determine the contributions of IL-35 to the Th2 responses. It is likely that the highly elevated miR-21 levels with the transfer of Bcl6-deficient Tregs in the Th2 model leads to reduced IL-35 levels, that further promotes exacerbated Th2 inflammation *in vivo*. This hypothesis can be validated by experiments testing miR-21/*Il12a* double knockout mice in the airway inflammation model.

Taken together, these experiments will help delineate a novel regulator of IL-35 and assess the relevance of miR-21/IL-35 regulatory circuitry in Treg control of Th2 inflammatory responses.

### **Assess signals that regulate miR-21 expression in Th2 inflammatory settings**

IL-6 acting *via* Stat3 induces miR-21 expression in inflammatory contexts (Iliopoulos *et al.*, 2010; Loffler *et al.*, 2007). However, a large number of other cytokines produced during inflammation can also activate Stat3. A critical question is whether there is one specific cytokine that directly promotes miR-21 transcription and up-regulation during inflammation, or if miR-21 is induced by multiple cytokines and other factors. Our results have indicated that wild-type Tregs transferred to Bcl6-deficient mice up-regulate miR-21. However, *in vitro* studies following treatment of wild-type Tregs with cytokines abundantly present in the Bcl6-deficient Th2 inflammatory environment (IL-4, IL-6 or a combination of IL-4 and IL-6) fail to induce miR-21 (data not shown). Thus, identifying the inflammatory signal/signals that turn on miR-21 following transfer of wild-type Tregs into Bcl6-deficient mice will provide useful insights into the upstream factors that regulate this miRNA in Th2 disease settings. Specifically, we have noted increased miR-21 expression in epithelial cells in our Th2 model following transfer of Bcl6-deficient Tregs. Thus, testing if epithelial cell-derived cytokines (particularly, thymic stromal lymphopoietin, TSLP) induce miR-21 in Th2 disease

settings will expand our understanding of the signaling networks in allergic diseases.

**Assess if the counter-regulation of miR-21 by Bcl6 and Stat3 is a critical node in Th2 differentiation program**

Stat3 positively regulates the miR-21 gene and the Stat3 DNA binding site is similar to a Bcl6 binding site (Shaffer *et al.*, 2000). Further, we have identified miR-21 as a novel Bcl6 gene target in T cells. Reporter assays demonstrate that Bcl6 and Stat3 oppose each other in the regulation of miR-21 by IL-6. Stat3 is required for complete Th2 differentiation, thus reduced Th2 differentiation of Stat3-deficient T cells could be due to their failure to induce miR-21. Thus, Bcl6 and Stat3 may counter-regulate Th2 differentiation via miR-21; with Bcl6 repressing miR-21 and thus inhibiting Th2 differentiation and Stat3 activating miR-21 and thus augmenting Th2 responses. Mating T cell-specific Stat3 conditional KO with miR-21 Tg mice and assessing if increased miR-21 can rescue the defective Th2 differentiation of Stat3-deficient T cells can test this hypothesis. Similarly, mating T cell-specific Bcl6 conditional KO to miR-21 KO mice will determine if deleting miR-21 can reduce the increased Th2 responses promoted by Bcl6-deficient T cells. Alternatively, these experiments can also be performed using miR-21 expressing retroviruses (miR-21 RV) in Stat3-deficient T cells and miR-21 inhibitors (antagomiR-21) in Bcl6-deficient T cells to assess the effect on Th2 differentiation.

## **Assess if miR-21 is specifically increased in Th2 pathologies in mice and human**

Here we will address the specificity of miR-21 expression in different types of inflammation. We will induce Th1-type and Th2-type lung inflammation in mice by LPS titration in our airway inflammation model. High dose LPS favors a Th1 response and low dose LPS promotes Th2 response (Eisenbarth *et al.*, 2002). We expect that miR-21 will be expressed higher in the lungs of mice in the Th2 airway inflammation than in the Th1 model. We could also analyze miR-21 expression in mouse models of Th17 inflammation. Our results on human patients with eosinophilic esophagitis and asthma have demonstrated elevated miR-21 in these human Th2 diseases. Similarly, we have also noted increased circulating miR-21 in these Th2 pathologies, thus indicating that miR-21 can serve as a non-invasive biomarker for human Th2 diseases. It will be interesting to extend these findings in other human Th2 conditions – allergic rhinitis, food allergies and atopic dermatitis. These studies will provide information on the association of miR-21 with atopy. In addition, while we did not detect miR-21 induction in biopsies of human patients with Crohn's disease (CD) associated with Th1 inflammation, it will also be important to assess miR-21 levels in the sera of the CD patients. These experiments in additional Th1 and Th2 disease models and human pathologies will provide extensive validation on using miR-21 as a clinical biomarker for Th2 diseases.

## **Assess the relevance of Treg defects to the myocarditis and pulmonary vasculitis that develops in Bcl6-deficient mice**

Bcl6-deficient mice develop spontaneous autoimmune disease in the heart and lungs. Considering that the disease is T cell-dependent, spontaneous and resembles the inflammation in mice with Treg defects, we proposed that defective Treg suppressor function in the absence of Bcl6 contributes to the pathology noted in Bcl6-deficient mice. While we do note Tregs with a Th2 bias in the heart and lungs of Bcl6-deficient mice which can offset the regulatory balance in these organs and mediate the development of Th2 inflammation, we were unable to address whether Bcl6-expressing Tregs traffic to these organs due to lack of appropriate tools. More recently, two different groups have developed Bcl6 reporter mouse strains (Kitano *et al.*, 2011; Liu *et al.*, 2012). It would be interesting to mate these Bcl6 reporter mice on the wild-type Foxp3gfp knock-in background, thus enabling tracking of the Bcl6-expressing Tregs to the inflamed heart and lungs following induction of experimental myocarditis (to assess recruitment to heart) or allergic inflammation (to assess recruitment to lungs). Further, this Treg-specific Bcl6 reporter mouse strain can also help to definitively assess the proportion of Tregs expressing Bcl6 in the total Treg subset and could be useful to determine if functionally distinct Treg subsets exist, such that Bcl6<sup>hi</sup> Tregs are the 'Tfr' Tregs that control the GC response and Bcl6<sup>int/low</sup> Tregs are involved in control of Th1, Th2 and Th17 cell responses, respectively.

### **Assess the importance of Tfr cells in the control of allergic inflammation**

T follicular regulatory T cells develop in a Bcl6-dependent manner in response to T-dependent antigens, to limit the size of Tfh and GC B cells in an immune response. Published literature assessing the functional relevance of these cells in regulating the quality and quantity of humoral immunity have relied on indirect means, such as fetal liver chimeras or adoptive transfer experiments (Chung *et al.*, 2011; Linterman *et al.*, 2011). We have generated the Treg-specific Bcl6 conditional knockout mice. These mice will be ideal tool to assess the importance of Tfr cells in control of allergic responses, Allergic reactions are caused by the exacerbations associated with uncontrolled IgE and the subsequent triggering of inflammatory mediators from mast cells and basophils, that mediate vasodilation, smooth muscle contraction and in some cases, IgE-mediated hypersensitivity. Thus, Tfr cells may play a cardinal role in controlling the IgE responses by limiting the GC reaction in the local lymphoid organs. Thus, allergic airway inflammation can be induced in cohorts of Bcl6<sup>fl/fl</sup>Cre<sup>+</sup> and Bcl6<sup>fl/fl</sup>Cre<sup>-</sup> mice and development of Tfh, GC B and plasma cell responses in the lung mediastinal lymph nodes can be assessed. This system will also be ideal to assess the Foxp3<sup>+</sup> Treg phenotype and function in the allergic immunity setting. Thus, these studies can provide information about the relative contributions of Foxp3<sup>+</sup> Tregs in control of Th2 immune responses and Bcl6<sup>+</sup>Foxp3<sup>+</sup>Tregs in regulation of the germinal center reaction and subsequent humoral immunity.



### **Assess the importance of Tfr cells in the control of flu infections**

Understanding the regulation afforded by Tfr cells is particularly important in the context of viral infections that can lead to the identification of useful strategies for effective vaccine development. Increased HIV GAG-specific Tfh cells, GC B cells and dysregulated antibody response (increased IgG1 subtype) has been reported in chronic HIV infections (Lindqvist *et al.*, 2012). Increased IFN $\gamma$ -producing Tfh cells have been reported following flu infection (Boyden *et al.*, 2012). The Treg-specific Bcl6 conditional mutant mice will serve as ideal tools to assess the importance of Tfr cells in regulating the Tfh and GC B response in viral infections. Here again, cohorts of Bcl6<sup>fl/fl</sup>Cre<sup>+</sup> and Bcl6<sup>fl/fl</sup>Cre<sup>-</sup> mice will be immunized with sublethal doses of influenza PR8 virus and we would assess the kinetic development of germinal centers, TFH responses, anti-flu IgM, anti-flu IgG, anti-flu IgA and anti-flu AFC. In addition, the ability of Bcl6<sup>fl/fl</sup>Cre<sup>+</sup> mice to resist secondary infection with flu virus and mount produce protective Ab responses can also be analyzed.

## **Assess the effect of Bcl6 in the Treg transcriptional landscape conferred by Foxp3-Gata3 interactions**

Treg cells express higher levels of Gata3 than conventional T cells and recent reports have identified that Gata3 expression in Tregs is important for Treg homeostasis and suppressor function (Wang *et al.*, 2011; Wohlfert *et al.*, 2011). Gata3 has been mapped as a Foxp3-interacting partner in Treg cells, with each factor augmenting the expression of the other factor (Rudra *et al.*, 2012). In addition, a subset of Foxp3-occupied genes in Tregs are co-bound by Gata3 (Rudra *et al.*, 2012). Thus, in the absence of Foxp3-Gata3 regulatory circuitry in Tregs, Gata3<sup>fl/fl</sup>Foxp3-YFP-Cre mice develop intestinal pathology and dermatitis accompanied with increase in IL-4<sup>+</sup>, IL-5<sup>+</sup>, IL-13<sup>+</sup> and Gata3<sup>+</sup> Th2 cells. Thus, the Gata3-Foxp3 complex in Tregs is critical for Treg control of Th2 responses (Rudra *et al.*, 2012). Bcl6 represses Gata3 expression and function and in the absence of Bcl6, Tregs fail to suppress Th2 inflammation. In addition, we have noted increased Foxp3 and Treg suppressor genes in Bcl6-deficient Tregs. Thus, it will be interesting to assess the effect of Bcl6 in the Foxp3-Gata3 subsection of the Treg transcriptome. Thus, we could perform biochemical analysis on Foxp3-Gata3 protein complexes isolated from Bcl6<sup>fl/fl</sup>Cre<sup>+</sup> and Bcl6<sup>fl/fl</sup>Cre<sup>-</sup> Tregs. This study may provide a comprehensive analysis of the regulation of Treg transcriptional landscape by Gata3 and Bcl6 that are both involved in Treg control of Th2 responses.

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## CURRICULUM VITAE

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### **Education**

2005	<b>B.Sc.</b> Biotechnology Mumbai University	Mumbai, India
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2012	<b>Ph.D.</b> Microbiology & Immunology Indiana University	Indianapolis, IN

### **Honors, Awards, and Fellowships**

2008	IUPUI Travel Award from the Graduate Student Organization for oral presentation at the Autumn Immunology Conference
2009	IUPUI Travel Award from the Graduate Student Organization for oral presentation at the Autumn Immunology Conference
2010	Pre-doctoral Fellowship Award from American Heart Association (AHA) (10PRE4620001)
2011	AAI Trainee Abstract Award to give an oral presentation at the 98th American Association of Immunologist Annual Meeting
2012	Travel Award to give an oral presentation at the 11th Annual Meeting of the Federation of Clinical Immunology Societies

### **Abstracts Presented and Conferences Attended**

2008	Deepali Sawant and Alexander Dent. Molecular mechanisms underlying FOXP3 promoter regulation by BCL6 and GATA3. Oral and poster presentation at Autumn Immunology Conference.
2009	Deepali Sawant and Alexander Dent. Control of Regulatory T cell activity by BCL6. Oral and poster presentation at Autumn Immunology Conference.
2011	Deepali Sawant, Sarita Sehra, Mark H. Kaplan, Alexander L. Dent. BCL6 controls the Th2 Inflammatory Activity of Regulatory T cells. Oral and poster presentation at American Association of Immunologists.

- 2011 Deepali Sawant, Sarita Sehra, Mark H. Kaplan, Alexander L. Dent. BCL6 controls the Th2 Inflammatory Activity of Regulatory T cells. Oral presentation at Federation of Clinical Immunological Societies.
- 2011 Deepali Sawant, Sarita Sehra, Mark H. Kaplan, Alexander L. Dent. The Transcriptional Repressor Bcl6 controls the Th2 Inflammatory Activity of Regulatory T Cells. Poster presentation at Sixth Annual NIH National Graduate Student Research Conference.
- 2012 Deepali Sawant, Sarita Sehra, Mark H. Kaplan, Alexander L. Dent. The Transcriptional Repressor BCL6 Controls the Th2 Inflammatory Activity of Regulatory T cells. Poster presentation at Keystone Symposia Conference: The Biology of Cytokines.

### **Peer Reviewed Publications**

- 2009 Kusam S, Munugalavadla V, Sawant D, Dent A. BCL6 cooperates with CD40 stimulation and loss of p53 function to rapidly transform primary B cells. *International Journal of Cancer*. 125(4):977-981.
- 2010 Mondal A\*, Sawant D\*, Dent AL. Transcriptional repressor BCL6 controls Th17 responses by controlling gene expression in both T cells and macrophages. *Journal of Immunology*. 184(8):4123-4132.  
\* These authors contributed equally.
- 2012 Sawant DV, Sehra S, Nguyen ET, Jadhav R, Englert K, Shinnakasu R, Hangoc G, Broxmeyer HE, Nakayama T, Perumal NB, Kaplan MH, Dent AL. Bcl6 Controls the Th2 Inflammatory Activity of Regulatory T Cells by Repressing Gata3 Function. *Journal of Immunology*, 189(10):4759-4769.  
Highlighted as Featured Article in "In This Issue" section.
- Sawant D, Wu H, Kaplan MH, Dent AL. The Bcl6 Target Gene MicroRNA-21 Promotes Th2 Differentiation by a T Cell Intrinsic Pathway. In revision, *Molecular Immunology*.
- Sawant D, Yao W, Tepper RS, Gupta SK, Kaplan MH, Dent AL. MicroRNA-21 Is Elevated in the Sera of Pediatric Eosinophilic Esophagitis and Pediatric Asthma Patients. In preparation.
- Sawant D, Yao W, Kaplan MH, Dent AL. Bcl6 Is Required to Maintain Regulatory T cell Stability and Repress the Development of Th2-like Ex-Regulatory T cells. In preparation.
- Hua L, Yao S, Pham D, Jiang L, Wright J, Sawant D, Dent AL, Braciale TJ, Kaplan MH and Sun J. Type I IFNs and IL-2 coordinate the *in vivo* development of cytotoxic T helper cells. In preparation.